Endocrine Effects on Mouse Oocyte DNA Methylation during Antral Follicle Development

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PhD

UNIVERSITY OF EDINBURGH

2005

Declaration

I declare that this thesis has been composed by myself and has not been submitted for any previous degree. The work described herein is my own and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the course of these studies.

Anna K. E. Swales

Abstract

Oocyte maturation has been the subject of extensive studies but there remains debate as to the effect of steroid hormones on this process and the subsequent implications for developmental competence. During oocyte maturation the establishment of the correct maternal DNA methylation pattern is vital if normal embryo development is to occur after fertilisation. Thus, any factors which perturb the process of DNA methylation could have an impact on the acquisition of oocyte developmental competence. This study investigates the effects of androgens and oestrogens on oocyte maturation, with particular emphasis on DNA methylation.

Alterations in DNA methylation and the subsequent expression of imprinted genes could be mediated through changes in ovarian expression of genes affecting the methylation process, such as one of the *Dnmt* or *Mbd* genes. However, no consistent, reproducible changes in expression levels of *Dnmt* or *Mbd* genes were observed after the administration of exogenous gonadotrophins. Further analysis found that exposure to raised steroid levels in culture had a significant effect on the global DNA methylation levels of oocytes. Although, no alterations in gene expression were previously identified this did not rule out the possibility that the observed changes to DNA methylation levels were the result of an effect on Dnmt proteins. To begin investigating this, the localisation of Dnmt1o was assessed after exposure to high levels of androgens and oestrogens. Under physiological conditions the Dnmt1o protein is present in the nucleus in growing oocytes and translocates to the cytoplasm as maturation progresses. Steroid exposure was found to alter the

localisation profile of Dnmt1o, making this a candidate mechanism by which the global DNA methylation levels are influenced by raised androgens and oestrogens.

The mechanisms controlling genomic imprinting during oocyte and embryo development are still being investigated. An increased understanding of genomic imprinting during development has been achieved through analysis of the gametes, embryos and offspring of mice with null mutations for *Dnmt* or *Mbd* genes. Although, *Mbd2*$^{-/-}$ mice have been reported to have a reduced litter size, the cause of this maternal effect is currently unknown. This study did not find any significant difference between maternal genotypes in the number of oocytes ovulated, fertilisation rate or percentage of 2-cell embryos developing to the blastocyst stage during IVF. Thus, the observed phenotype of small litter size may not be the result of impaired oocyte maturation or early embryo development occurring in the absence of Mbd2.

The increasing use of assisted reproductive techniques means that a full understanding of the role of the environment on oocyte developmental competence is vital. This thesis presents evidence that raising the concentration of androgens and oestrogens can influence the process of DNA methylation during oocyte maturation.

Publications arising, or containing work from this thesis

Published Paper

Swales A.K.E. and Spears N. (2005)

Genomic Imprinting and Reproduction

Reproduction, **130**(4):389-99

Submitted paper

Murray A.A., Swales A.K.E., Smith R.E., Allison V., Hillier S.G. and Spears N.

The Effect of Altered Gonadotrophin and Steroid Levels on Murine Follicular Development and Oocyte Competence

Molecular Human Reproduction

Presentations of work contained within this thesis

Platform presentations

Swales A.K.E. and Spears N.

Mouse oocytes exposed to raised androgen and oestrogen levels in vitro exhibit increased DNA methylation.

National Ovarian Workshop, Edinburgh 2004, Session prize

Anna K.E. Swales and Norah Spears

Mouse Oocytes Exposed to Raised Androgen and Oestrogen Levels in vitro Exhibit Increased DNA Methylation

Biology of Reproduction, 37th Annual Meeting, Vancouver 2004

Poster presentations

A.K.E. Swales and N. Spears

Mouse oocytes exposed to raised androgen and oestrogen levels in vitro exhibit increased DNA methylation.

XVth Ovarian Workshop, Vancouver 2004

Assistance given throughout this investigation

The work detailed in this thesis was conducted by myself with the following exceptions:

Chapter 3: Ian Simpson designed the RT-PCR primers for *Dnmt3b* and Donncha Dunican designed the *Mbd1-3* and *MeCP2* primers. Rowena Smith carried out the RT-PCR reactions for the *Mbd* genes and assisted in optimisation of several of the RT-PCR protocols.

Chapter 4: Nathalie Beaujean taught me the methylation immunohistochemistry protocol. Linda Wilson carried out the initial confocal microscopy and provided training to allow me to obtain images independently.

Chapter 6: Superovulation injections and assistance with the IVF procedure were carried out by Alison Murray and Rowena Smith.

I received assistance in setting up large follicle cultures from several laboratory members. They were: Vivian Allison, Alison Murray and Rowena Smith.

Acknowledgements

First and foremost I would like to thank Norah Spears for all the supervision and support she provided throughout my studies. Thanks also to my second supervisor, Richard Meehan and my thesis committee, Philippa Saunders, Brian Hendrich and Peter Kind, who all provided valuable discussions through the progression of the PhD.

I am also indebted to all members of the Spears Group (both past and present) for their assistance, advice and friendship throughout my time in the lab. Special thanks to Alison Murray and Rowena Smith for all their help over and above the call of duty! Also, Mike Molinek and Vivian Allison for teaching me the all important Scottish phrases to go alongside the science. Technical support and guidance have also been provided by people outside of our lab, I am grateful for the assistance provided by Grace Grant, Nathalie Beaujean, Linda Wilson, Allison Wroe and Duncan McNeil. I'd also like to thank the Peet Foundation for providing funding for my laptop, which made the writing process so much easier. Cheers to all the other students, especially Tammy, Natasha, Mark and Dennis, for reminding me that there should be more to life than just work. Special thanks to both Ali and Jane McNeilly for indulging my caffeine cravings and always being there for me.

A big thanks has to go to all those people who although non-scientists had to endure all the ups, downs and details of my research – Karen and Chris thanks for putting up with me. Finally, a huge thank you has to go to my family for their extensive encouragement and always believing I'd get there – I've really appreciated your support over the last 4 years.

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Abbreviations

$\alpha\beta ERKO^{-/-}$	alpha and beta oestrogen receptor knockout
α-MEM	alpha Minimum Essential Medium
Amh	Anti-mullerian hormone
ANOVA	Analysis of Variance
AR	Androgen Receptor
ArKO	Aromatase Knockout
ART	Assisted Reproductive Technology
AS	Angelman Syndrome
Bax	Bcl2-associated X protein
Bcl-x	B-cell leukemia/lymphoma 2
BERKO	oestrogen receptor beta knockout
Bmp	Bone morphogenetic protein
BS	Blocking Solution
BSA	Bovine Serum Albumin
BWS	Beckwith-Wiedemann Syndrome
Ca^{2+}	Calcium
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
c-fos	*fos proto-oncogene*
CH_3	Methyl Group
CL	Corpus Luteum
c-mos	Cytostatic factor involving *mos* kinase
COC	Cumulus-Oocyte complex
cox-2	cyclooxygenase 2
CSF	Cytostatic Factor
cx	connexin protein
dazl	*deleted in azoospermia like*
DES	Diethylstilboestrol
DHT	Dihydrotestosterone
DMR	Differentially Methylated Regions

DNA	Deoxyribonucleic Acid
Dnmt	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
E	Embryonic Day
EGF	Epidermal growth factor
EGF-like	Epidermal growth factor-like
ER	oestrogen receptor
ERKO	oestrogen receptor alpha knockout
ETOH	Ethanol
FF-MAS	Meiosis Activating Sterol from Follicular Fluid
FGF	Fibroblast growth factor
Figla	*factor in the germline alpha*
FSH	Follicle Stimulating Hormone
FSHR	Follicle Stimulating Hormone Receptor
Gas-6	*Growth Arrest-Specific gene-6*
GC	Granulosa Cell
Gdf-9	Growth differentiation factor-9
Gpr3	G-protein-coupled receptor-3
Gnas	*guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide gene*
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
Has2	*hyaluron synthase-2*
hCG	Human Chorionic Gonadotrophin
HDAC	Histone Deacetylase
IAP	Intracisternal A Particle
ICF	Immunodeficiency, centromeric region instability, and facial anomalies syndrome
ICM	Inner Cell Mass
ICSI	Intra Cytoplasmic Sperm Injection
Igf	Insulin-like growth factor
IGF-1R	Insulin-like growth factor-1 receptor

Il	Interleukin
IP3	Inositol 1,4,5-triphosphate
IU	International Unit
IVF	*in vitro* Fertilisation
KL	Kit ligand
KO	Knockout
LH	Luteinising Hormone
LHR	Luteinising Hormone Receptor
LIF	Leukemia Inhibitory Factor
LOS	Large Offspring Syndrome
MAPK	Mitogen Activated Protein Kinases
Mbd	Methyl Binding Domain
MDBP	Methylated DNA Binding Protein
MPF	Maturation Promoting Factor
mRNA	Messenger Ribonucleic Acid
Ms-SNuPE	Methylation-sensitive single nucleotide primer extension
MTOCs	Microtubule organising centres
NSN	Non-Surrounded Nucleolus
NuRD	Nucleosome Remodelling and Histone Deacetylation Complex
PBS	Phosphate Buffered Solution
PCNA	Proliferating cell nuclear antigen
PCOS	Polycystic Ovarian Syndrome
PCR	Polymerase Chain Reaction
PDE3A	phosphodiesterase 3A, cGMP-inhibited
Peg	*Paternally expressed gene*
PFA	Paraformaldehyde
PG	Prostaglandin
PGC	Primordial Germ Cell
PI	Propidium Iodide
Pin	protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting gene
PLC	Phospholipase C
PMSG	Pregnant Mare Serum Gonadotrophin

PMT	Photomultiplier Tube
PWS	Prader-Willi Syndrome
RNA	Ribonucleic Acid
SEM	Standard Error of the Mean
SF-1	Steroidogenic Factor-1
SN	Surrounded Nucleolus
sox	*sry (sex determining region Y)-box gene*
StAR	Steroidogenic acute regulatory protein
TF	Transcription Factor
TNF	Tumour necrosis factor
Trk	Tyrosine Kinase
Tsg-6	Tumour necrosis factor alpha-stimulated gene 6 protein
Wnt	*Wingless-type MMTV integration site gene*
ZP	Zona Pellucida

Chapter 1

General Introduction

1.1 INTRODUCTION

Although there have been extensive studies investigating the role of steroids on follicular development and on aspects of oocyte maturation, the effects of these hormones on the developmental competence of the oocyte are still under debate. The importance of furthering our understanding of the mechanisms of oocyte maturation and the factors that influence this becomes apparent when Assisted Reproductive Techniques (ARTs) are considered. The use of these techniques is becoming increasingly common but, as the relatively low success rates demonstrate, many aspects of the technologies are yet to be fully investigated. One area of interest is epigenetics, particularly genomic imprinting, with recent studies finding a correlation between assisted conceptions and an increase in the incidence of normally rare imprinting disorders. One of the major differences between embryos obtained from either natural or assisted conceptions is in the steroidal environment both they, and the gametes which gave rise to the embryos, were exposed to. This variation in steroidal milieu may occur *in vivo* due to superovulation or be the result of *in vitro* culture systems (Kajta, 1998). This study aims to investigate the effects of androgens and oestrogens on mammalian oocyte maturation, with particular emphasis on genomic imprinting. The term "androgens" is used within this thesis to encompass the group of androgenic hormones that act through the classical androgen receptors. Likewise, the term "oestrogens" is used to cover a range of oestrogenic hormones that act through classical oestrogen receptors, including oestradiol and oestrone. The research model utilised in this project is the murine system, with all work referring to this species unless otherwise stated.

1.2 PRIMORDIAL GERM CELLS (PGCs)

1.2.1 Localisation, Proliferation and Migration of PGCs

Early mouse embryos contain an initial population of PGCs which will go on to form the oocytes and spermatozoa of the adult gonads. These PGCs are initially identified near the posterior region of the primitive streak, from approximately embryonic day 7.5 (E7.5), by the presence of alkaline phosphatase activity (Chiquoine, 1954; reviewed by Matsui & Okamura, 2005). They are localised within the extra-embryonic mesoderm resulting in them being separated from the embryonic somatic cells and protected from many of their differentiation signals. As any epiblast cells that are transplanted to the proximal epiblast are capable of forming PGCs, there may not be a subset of cells which are pre-programmed to form the germ line. It may be that germ cell determination is caused by local tissue signals (Figure 1.1) (Tam & Zhou, 1996; reviewed by Matsui & Okamura, 2005). Bone morphogenetic protein 4 (Bmp4) is proposed as one such local signal. Mice homozygous for a functional mutation of *Bmp4* (*Bmp4*$^{-/-}$) do not have any PGCs while the heterozygote (*Bmp4*$^{+/-}$) has a smaller PGC founder population, demonstrating that Bmp4 is vital for the germ cell determination (Lawson *et al.*, 1999). PGCs undergo mitotic proliferation during their migration to the gonadal ridge (reviewed by Molyneaux & Wylie, 2004). This migration into the embryonic mesoderm and through the hindgut begins at E8.5, with the binding of Kit ligand (KL) to the c-Kit receptor being involved in mediating the required interactions between somatic and germ cells (Pesce *et al.*, 1997). They reach the undifferentiated gonadal ridge at E10.5-11.5 but proliferation continues until E12.5-13.5 (reviewed by De Felici *et al.*, 2005). The rate of this proliferation seems to be determined by Pin1 with a species specific number of divisions

occurring (Atchison *et al.*, 2003). From E12.5, the gonads become sexually dimorphic and subsequently impose a sex determined fate on the PGCs residing within the developing gonad (reviewed by McClaren, 2003). PGCs located in a developing ovary cease to replicate by mitosis and enter meiosis to become oocytes while those in the early testes enter mitotic arrest and are termed prospermatogonia (Hilscher *et al.*, 1974; as reviewed by De Felici *et al.*, 2005). In addition to factors that control the migration and determination of the PGCs they are also dependent *in vivo* on KL for survival, while *in vitro* studies also suggest a survival role for leukaemia inhibitory factor (LIF), growth arrest-specific protein-6 (Gas-6) and interleukin-4 (Il-4) (Godin *et al.*, 1991; Matsui *et al.*, 1991; Matsubara *et al.*, 1996; Cooke *et al.*, 1996). In culture, PGC proliferation is stimulated by tumour necrosis factor–α (TNF-α), retinoic acid and cyclic adenosine monophosphate (cAMP) although whether these factors carry out a similar role *in vivo* is yet to be determined (Kawase *et al.*, 1994; Koshimizu *et al.*, 1995; De Felici *et al.*, 1993). Around E13.5 there is increased apoptosis of male PGCs, while an increase in female germ cell death occurs nearer E15.5 (Wang *et al.*, 1998; Ratts *et al.*, 1995; as reviewed by De Felici *et al.*, 2005). During these periods of germ cell reduction B-Cell Leukemia/lymphoma 2 (Bcl-x) is responsible for protecting germ cells from apoptosis whilst the presence of Bcl2-associated X protein (Bax) increases cell death (Rucker *et al.*, 2000).

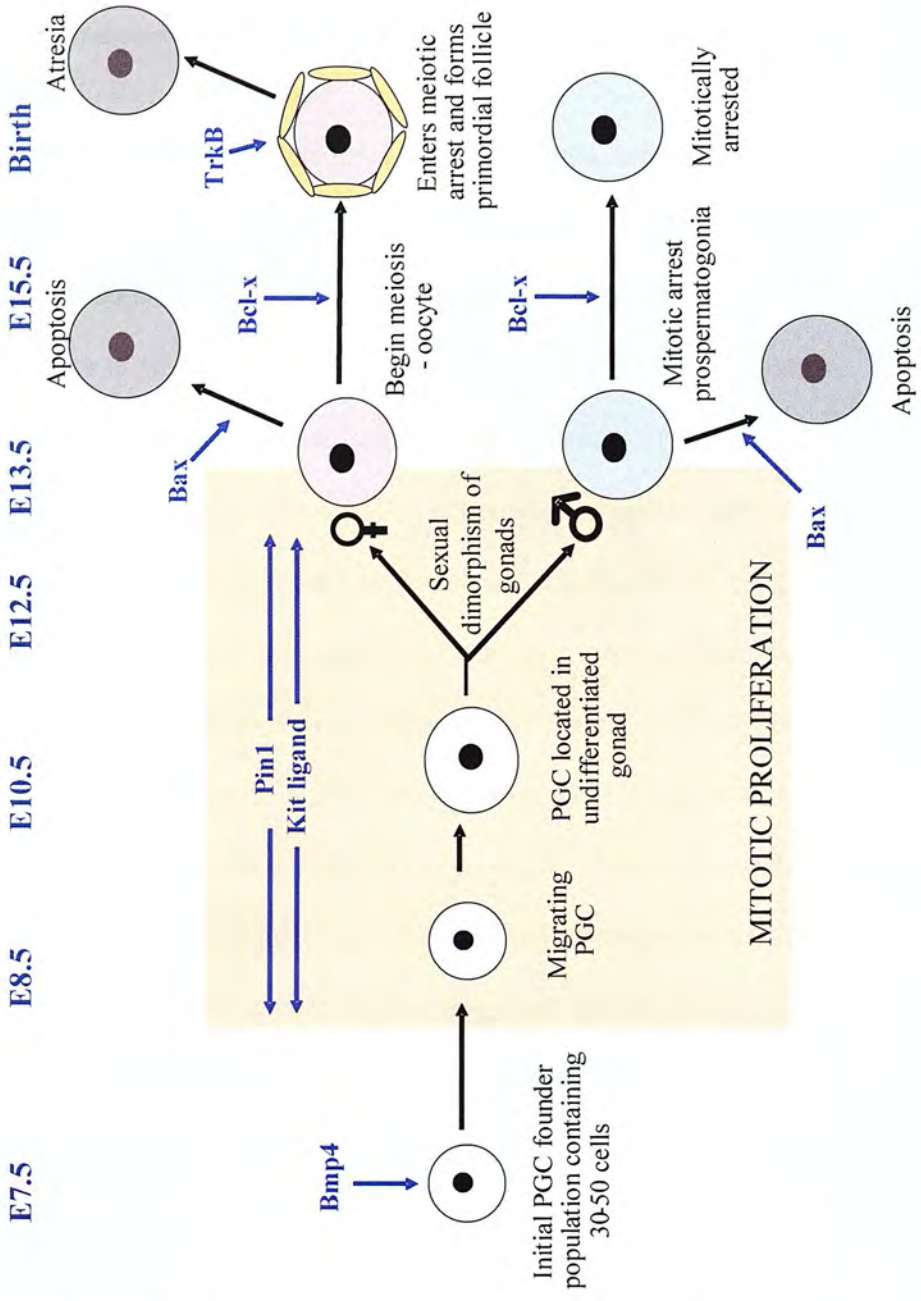


Figure 1.1:- Diagram illustrating the timecourse and main factors involved in primordial germ cell formation and migration. Bax – Bcl-2-associated X protein; Bcl-x – B-Cell Leukemia/lymphoma 2; Bmp4 – Bone morphogenetic protein-4; E - Embryonic day; PGC – Primordial germ cell; Pin1 - protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting gene; TrkB - Tyrosine Kinase B.

1.3 FOLLICLE DEVELOPMENT (Figure 1.2)

1.3.1 Primordial Follicle Formation

Once located in the ovary, PGC clusters must be broken down: the oogonia interact with surrounding somatic cells to form primordial follicles (reviewed by Skinner 2005). Each primordial follicle consists of a quiescent oocyte in meiotic arrest surrounded by a layer of flattened granulosa cells (GCs). Oogonia which do not successfully become part of a primordial follicle cannot survive. The exact timing of primordial follicle formation is species-specific, occurring during fetal development in primates while in rodents this process is underway around the time of birth. When no oocytes are present in the female gonad the somatic cells tend to differentiate towards a male phenotype, with structures reminiscent of seminiferous tubules or Sertoli cells developing (Hashimoto *et al.*, 1990, Mclaren, 1991; Guigon *et al.*, 2005). Although this suggests that the oocyte has an active role in determining gonad differentiation, the study of mice lacking functional oestrogen receptors α and β (*ERαβ*$^{-/-}$) and homozygous null mutants for the aromatase enzyme (ArKO$^{-/-}$) mice suggest that it is the action of oestrogen, rather than the presence of oocytes, which maintains the normal ovarian somatic cell phenotype (Couse *et al.*, 1999; Britt *et al.*, 2001; Britt & Findlay, 2003). Additionally, in the ovary of adult mice lacking the *deleted in azoospermia like* gene (*dazl*$^{-/-}$) there is no evidence of differentiation towards a testis phenotype, even though there are no oocytes present. Interestingly, the ovarian somatic cells in these animals maintain their steroidogenic function (McNeilly *et al.*, 2000). It is largely accepted that the population of primordial follicles represents the lifetime complement of female germ cells and is not renewable, although, the possibility of ovarian stem cells renewing the oocyte

population has been proposed (Johnson *et al.*, 2004). Several factors have been identified as playing important roles in oocyte survival and primordial follicle formation. A member of the tyrosine kinase (Trk) receptor family, TrkB, has been shown to effect germ cell and primordial follicle survival. If TrkB is inhibited at the time when primordial follicles are forming there is extensive germ cell death (Spears *et al.*, 2003). The expression profile of *factor in the germline-α* (*Figla*) and the phenotype of female mice with disrupted *Figla* expression (*Figla*$^{-/-}$) suggest a role for this protein in oocyte survival and primordial follicle formation (Soyal *et al.*, 2000). No primordial follicles form in the ovaries of *Figla*$^{-/-}$ females and widespread oocyte death is observed demonstrating a vital role of Figla in some aspect of germ cell survival and follicle formation (Soyal *et al.*, 2000). A similar role of FIGLA in the human fetal ovary is suggested by the increase in *FIGLA* expression in parallel with the time of primordial follicle formation (Bayne *et al.*, 2004). The ovaries of *Wnt4*$^{-/-}$ females also lack primordial follicles and oocytes, suggesting that this gene may also have a role in determining the early organisation of the ovary and perhaps follicle development (Vainio *et al.*, 1999).

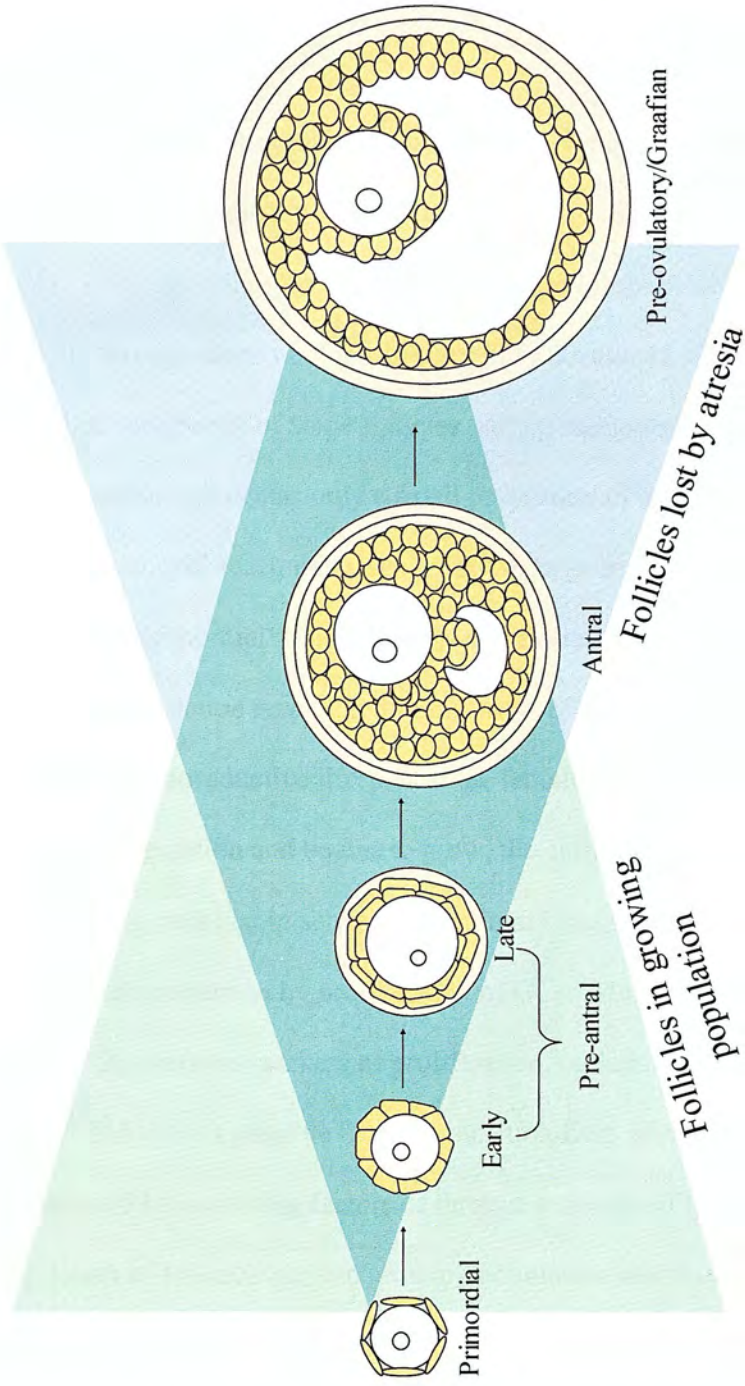


Figure 1.2:- Follicle development:- When the previously quiescent primordial follicle is recruited to the growing pool the granulosa cells become cuboidal and oocyte growth is initiated. During pre-antral development oocyte growth continues, granulosa cells proliferate and thecal layers form. The development of the antrum marks the start of the antral phase of development. As the antral cavity volume increases two sub-populations of granulosa cells form with cumulus cells surrounding the oocyte and mural granulosa cells at the outer edge of the antrum.

1.3.2 Primordial Follicle Recruitment

Once primordial follicle formation is complete, follicles from the quiescent pool begin to be recruited to form a population of growing follicles (reviewed by Fortune, 2003; Skinner, 2005). Follicles which are activated (or released from inhibition) prior to puberty will die through atresia before they are fully developed. Once puberty has been reached the growth of some follicles will be supported to the Graafian stages and ovulation will occur; only a small proportion of the follicles recruited to the growing pool will reach this stage, with the large majority becoming atretic. Tight regulation of primordial follicle recruitment is vital as the loss of follicles into the growing population results in diminishment of the follicular reserve and therefore determines the reproductive lifespan of the female. When a primordial follicle leaves the resting population and begins to grow, the earliest observable change is the GCs becoming cuboidal in shape. This primary stage of pre-antral follicular development is characterised by proliferation of GCs. Molecular analysis demonstrates that these GCs express markers of proliferation, including proliferating cell nuclear antigen (PCNA). It is possible that the transition from primordial to primary follicle is mediated by activating factors or through a release of inhibition. The spontaneous initiation of follicles into the growing population seen in cultured ovaries is suggestive of an inhibitory factor acting *in vivo*. Anti-Mullerian hormone (Amh) has an inhibitory effect on primordial follicle recruitment both *in vitro* and *in vivo* (reviewed by Fortune, 2003 and Skinner, 2005), with $Amh^{-/-}$ ovaries becoming depleted of primordial follicles more rapidly than in $Amh^{+/+}$ animals (Durlinger *et al.*, 1999; 2002). Additionally, there are many factors which although capable of influencing follicle recruitment *in vitro* there is as yet no evidence that they perform

this function *in vivo*. KL or fibroblast growth factor (FGF) in culture cause a reduction in the number of primordial follicles and an increase in pre-antral follicle numbers within the rat ovary, with the implication that they stimulate the primordial to primary transition (Parrott & Skinner, 1999; Nilsson *et al.*, 2001; reviewed by Skinner, 2005). This transition process is also seen to increase when LIF is added to the culture, which may be mediated through its ability to increase KL levels (Nilsson *et al.*, 2002; reviewed by Skinner, 2005). Insulin also has a stimulatory effect on primordial follicle transition and when added in combination with either LIF or KL there is an additive effect (Nilsson *et al.*, 2002; Parrot & Skinner 1999; Kezele *et al.*, 2002). Pre-antral follicle growth is not dependent on pituitary gonadotrophins, although primary follicles have detectable levels of follicle stimulating hormone receptor (*FSHR*) messenger ribonucleic acid (mRNA) this may not signify the presence of active FSHR (O'Shaughnessy *et al.*, 1996).

1.3.3 Pre-Antral Follicle Development

Once follicles have developed a second layer of GCs they are deemed to have entered the secondary phase of pre-antral growth (reviewed by Fortune, 2003). During this stage GC proliferation continues and the number of layers surrounding the oocyte increases (Figure 1.2). Primary pre-antral follicles require the oocyte specific growth differentiation factor 9 (Gdf-9) if they are to progress to the secondary stage (Dong *et al.*, 1996). *Gdf-9* is expressed in all oocytes except those quiescent oocytes in primordial follicles (McGrath *et al.*, 1995). During mid to late pre-antral stages, a layer of theca cells develops by recruiting surrounding somatic cells. KL is involved in the development of this theca layer, but whether its role is

recruitment or purely proliferation is undetermined (Parrott & Skinner, 1997; Parrott & Skinner, 2000). These early stages of follicle growth and development are able to occur independently of gonadotrophin stimulation, with luteinising hormone receptors (LHRs) and FSHRs beginning to be synthesised on the theca and GCs respectively during the late pre-antral stage (Camp *et al.*, 1991).

In mice, the oocyte contained within a pre-antral follicle enters a phase of rapid growth which is complete by the time the follicle reaches a diameter of approximately 150μm and prior to antrum formation (Carabatsos *et al.*, 1998). The oocyte must also store mRNA and proteins that will be vital for later stages of oocyte maturation, fertilisation and pre-implantation development. At this stage the oocyte is not competent to resume meiosis when isolated from its follicular environment. At least *in vitro,* oocyte growth is stimulated by KL acting through the c-Kit receptors which are present on the oocyte (Packer *et al.*, 1994). The oocyte also synthesises and secretes glycoproteins which form the zona pellucida (ZP) around the oocyte which prevents polyspermy and inappropriate implantation. The ZP consists of three glycoproteins, ZP1, 2 and 3 which require other factors including *figla* if they are to be correctly expressed (Epifano *et al.*, 1995; Liang *et al.*, 1997). The oocyte maintains communication and receives chemical substrates from the surrounding GCs via connexin (cx) -dependent gap junctions. Mice lacking functional *cx37* (*cx37*$^{-/-}$) have impaired oocyte-GC communication. This results in follicle development arresting at the time of antrum formation and incomplete oocyte maturation (Simon *et al.*, 1997). Follicles of *cx43*$^{-/-}$ animals have follicle development arrested even earlier, at the primary pre-antral stage, as it is required for

gap junction formation between neighbouring GCs (Ackert *et al.*, 2001). The presence of gap junctions allows vital cross-talk to coordinate development between the follicular cells and the oocyte (Eppig, 1991; Eppig, 2001; reviewed by Fair, 2003). The oocyte appears to have responsibility for determining the rate at which the follicle grows: when the quiescent oocyte of a primordial follicle is removed and replaced with a growing oocyte from a secondary pre-antral follicle (one with two layers of GCs), the earlier stage follicular cells grow more rapidly than controls until they match the developmental stage of the growing oocyte (Eppig *et al.*, 2002; reviewed by Matzuk *et al.*, 2002).

1.3.4 Antral Follicles

The end of the pre-antral development phase is marked by the formation of a fluid filled antral cavity between the GCs (Figure 1.2). Follicle growth continues in antral follicles due to the combination of GC proliferation and GC secretion of follicular fluid to increase the volume of the antral cavity. It is hypothesised that the antrum may allow a greater number of GCs to be supported, by providing nutrient support and hormonal stimulation in the absence of a blood supply (Rodgers *et al.*, 2001). The antrum also provides fluid which allows the efficient expulsion of the oocyte at ovulation. However, analysis of antral size suggests the volume of follicular fluid is in excess of that required purely for ovulation (Rodgers *et al.*, 2001). The *in vitro* and *in vivo* formation and maintenance of the antrum has been shown to be follicle stimulating hormone (FSH) dependent (Halpin *et al.*, 1986; Hartshorne *et al.*, 1994; Mitchell *et al.*, 2002). Over the period of antrum formation there is a separation of the GCs into two distinct populations, the mural GCs around the outer edge of the

antrum and the cumulus GCs which closely surround the oocyte. Cumulus cells will be ovulated with the oocyte and aid its expulsion from the follicle and its entry to the oviduct. The mural GCs have a role in follicular rupture at ovulation and undergo luteolysis to form much of the corpus luteum (CL), along with theca cells. Despite the presence of several locally derived factors, whose presence are beneficial to antral follicle growth and development, there is an absolute requirement for the pituitary gonadotrophins. Early stage (primary) follicles express the FSHR on the GCs and although more developed antral follicles have increased expression of these receptors, there is no change in their localisation (Camp *et al.*, 1991). *FSHR*$^{-/-}$ females are infertile with no mature follicles evident in the ovary (Dierich *et al.*, 1998). *Insulin-like growth factor-1* deficient (*Igf-1*$^{-/-}$) mice have severely reduced FSHR levels accompanied by arrested antral follicle development. This demonstrates that FSHR expression is stimulated by GC Igf-1 and that a suboptimal level of the FSHR is detrimental to follicular development (Zhou *et al.*, 1997). The acquisition of gonadotrophin dependence is a key selection stage in determining which follicles will continue along the developmental pathway and potentially ovulate. Follicles which do not have adequate levels of FSHR or are not exposed to high enough levels of FSH will enter atresia and die. There is a positive feedback role of FSH on the expression of both its own receptor and the aromatase receptor of GCs, although FSHR is still present in hypogonadal mice with no circulating gonadotrophins (Zhou *et al.*, 1997; O'Shaughnessy *et al.*, 1994). LHRs are found on the theca cells of the follicle; later in development, in response to FSH, larger antral follicles have an increase in theca LHR and expression on the mural GCs (Erickson *et al.*, 1979; Camp *et al.*, 1991; Segaloff *et al.*, 1990). Paracrine signals from antral

stage oocytes are able to inhibit the expression of LHRs on both mural and cumulus GCs *in vitro*. This suggests it is an oocyte specific secretion *in vivo*, possibly Gdf-9, which prevents cumulus cells developing LHRs while the mural cells escape this short-range inhibitory signal (Eppig *et al.*, 1997; Elvin *et al.*, 1999). FSH and luteinising hormone (LH) are able to stimulate steroid production by the follicle; with theca cells mainly synthesising androgens under the influence of LH and FSH controlling GCs production of oestrogens (see Section 1.4). Antral stage oocytes are capable of resuming meiosis and when removed from the follicle spontaneously re-enter meiosis. Under normal conditions the oocyte is held in meiotic arrest until the ovulatory LH surge. If an oocyte is to remain in meiotic arrest it requires high cAMP levels. It is predicted that GC control of oocyte cAMP levels is mediated through the oocyte Gpr3 receptor; this receptor may mediate G_s protein activation which in turn stimulates adenylyl cyclase synthesis of cAMP (Mehlmann *et al.*, 2002; Kalinowski *et al.*, 2004; Mehlmann *et al.*, 2004).

1.3.5 Pre-ovulatory Follicles

During antral development, the follicles are gonadotrophin-dependent and a cohort containing several follicles will progress through antral development as long as there is a sufficiently high circulating level of FSH. The steroidogenic ability of the follicle increases with growth. This means that more developed follicles will synthesise greater concentrations of both oestradiol and androgens than less mature follicles. In addition to having local ovarian actions these steroids also pass into the circulation. When oestradiol levels reach a high concentration it exerts a negative feedback effect on FSH production in the pituitary, thus as oestradiol levels rise there

is a concurrent reduction in FSH synthesis and the level reaching the growing follicles. Increasing levels of inhibins also contribute to the reduction in FSH. This reduction results in inadequate gonadotrophin support of the less mature and still gonadotrophin-dependent follicles, which will then become atretic. It is through this mechanism that a species specific number of follicles become dominant, undergo pre-ovulatory development and ovulate. One feature of late antral development is the FSH-induced formation of LHRs on the GCs of the follicle. As the binding of FSH and LH to their appropriate receptors activates a common intracellular cAMP pathway, the effects of the gonadotrophins in pre-ovulatory follicles are considered to be additive. Thus, pre-ovulatory follicles are less dependent on FSH with their growth and development also being supported by LH.

1.4 STEROIDOGENESIS

Cholesterol is imported from the circulation into the theca cells of the follicle: the rate of importation and the level of the cholesterol side-chain cleavage enzyme P450scc present in the theca determine the rate at which cholesterol enters the steroidogenic pathway. Both of these factors are controlled by cAMP stimulated by LH, the cAMP-mediated up-regulation of the steroid hydroxylases depends upon steroidogenic factor 1 (SF-1). Cholesterol is moved to the inner mitochondrial membrane, a process which requires steroidogenic acute regulatory (StAR) protein, and then converted to pregnenolone (Kiriakidou *et al.*, 1996; Christenson & Devoto, 2003). The subsequent conversion of pregnenolone to progesterone is followed by the cytochrome P450c17α mediated synthesis of androgens, principally

androstenedione (Smyth *et al.*, 1993). These androgens then cross into the GCs where the androgen is aromatised to oestrogens, mainly oestradiol, by cytochrome p450 aromatase (Figure 1.3) (as reviewed by Magoffin, 2005). As GCs lack P450c17α they are unable to synthesise androgen to act as a substrate for oestradiol synthesis. The GCs are therefore dependent on theca-synthesised androgens, thus any factors which act to regulate thecal androgen production also impact on GC oestradiol output. Although the theca cells do not contain FSHR, FSH has been shown to increase both P450c17α mRNA expression and LH-responsive androgen synthesis. This suggests a mechanism whereby FSH causes stimulation of a GC paracrine signal which acts upon the theca cells. One such paracrine factor is inhibin A which is produced by GCs but acts to enhance LH-stimulated androgen production (Smyth *et al.*, 1993; reviewed by Wood & Strauss, 2002). FSH stimulates the expression of the inhibin α-subunit and the activin/inhibin β-subunits in cultured GCs, suggesting that the levels of these subunits increase as a follicle develops (Turner *et al.*, 1989). This relationship whereby the same stimulant of oestrogen synthesis by the GCs also causes paracrine signalling to increase androgen synthesis in the theca ensures that adequate substrate for aromatisation is supplied.

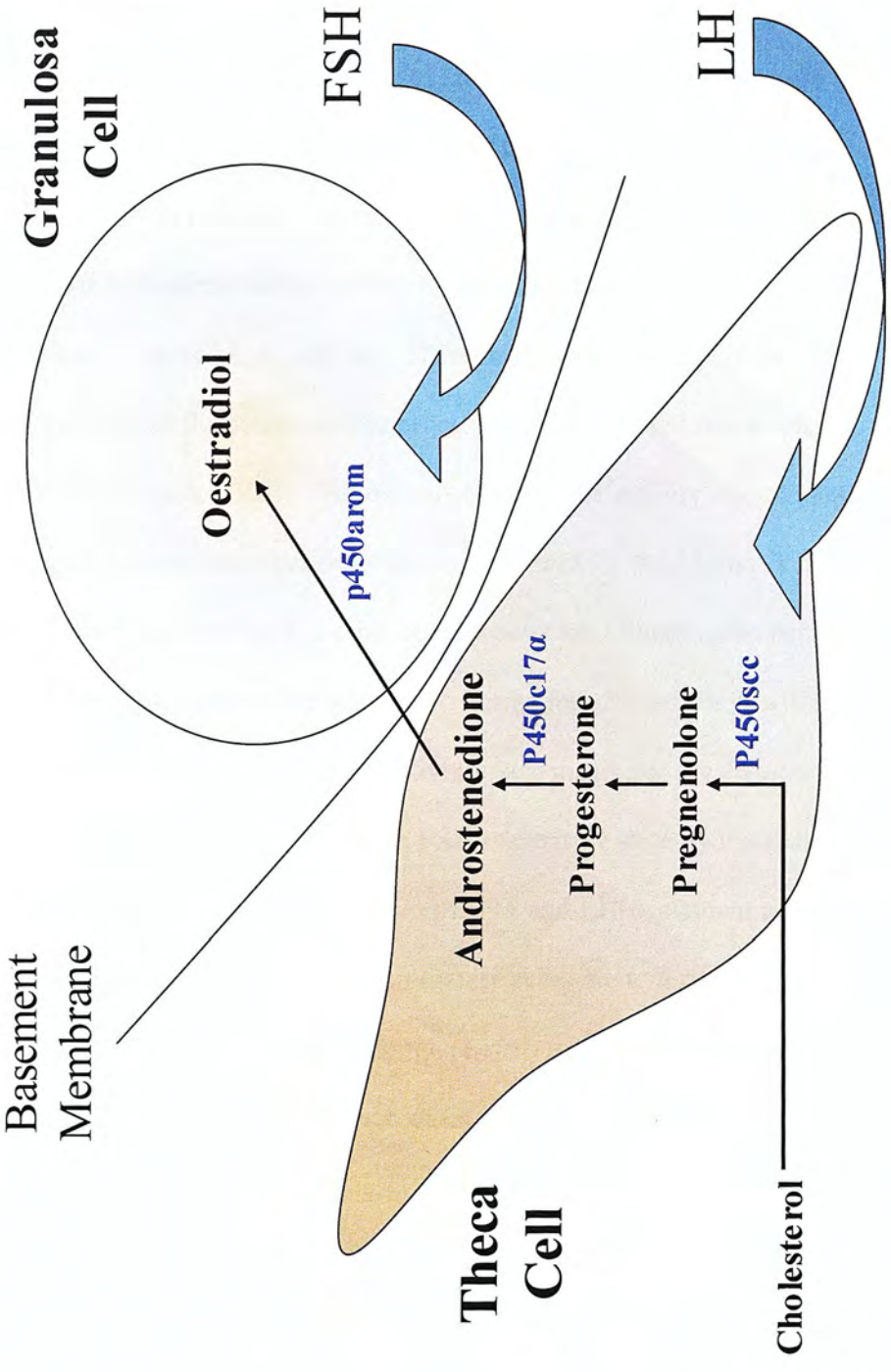


Figure 1.3:- Steroidogenic pathways. Androgens are synthesised in the theca cells and then pass into the granulosa cell where they are aromatised to oestrogens. LH-Luteinising hormone; FSH-Follicle stimulating hormone

1.4.1 Activins and Inhibins

Inhibin is a glycoprotein heterodimer made up of an α and a β-subunit. There are two forms of inhibin which have a common α-subunit but each has a different β-subunit. Inhibin A contains βA while the inhibin B heterodimer consists of a βB subunit. Activin is a homodimer of the inhibin β-subunits which can combine to give the three forms of activin, A, B and AB. There is a complex interplay of both the activins and inhibins on the steroidogenic processes of the GC and theca cells (as reviewed by Wood & Strauss, 2002). The activins enhance the activity of aromatase in the GC causing an increase in oestradiol output (Ying *et al.*, 1986; Hillier & Miro, 1993). Activin also acts to limit the LH-stimulated production of androgens from the theca and through this restriction of the substrate required for aromatisation will ultimately limit oestradiol production. High activin levels mean that the influence of FSH is enhanced, maintaining the follicle in an FSH-responsive state by increasing FSHRs. Inhibins cause an increase in P450c17α mRNA and LH-dependent androgen synthesis in the theca cells. This increase in aromatase substrate will result in an overall increase in GC oestradiol output, although *in vitro* inhibins have been shown to decrease FSH-mediated oestradiol secretion of GCs (Ying *et al.*, 1986).

Overall, smaller follicles produce activins but as they grow, and under the stimulatory influence of FSH, inhibin production increases. Dominant follicles have greater levels of the inhibins present, which in turn has a negative feedback effect on the pituitary to reduce further FSH secretions. This reduction in FSH level causes the smaller follicles within the ovary to express lower levels of βB subunit, reducing both their activin and inhibin levels. It is this reduction of FSH which results in

many subordinate follicles entering atresia as they are not sufficiently developed to withstand this decrease in gonadotrophin level.

1.5 THE EFFECTS OF OVARIAN STEROIDS

The presence of the steroid receptors within the ovarian follicle and the phenotypes of mice with homozygous null mutations of these receptors demonstrate the importance of steroidal hormones in female reproduction (Table 1.1). The examination of the roles of steroids within the ovary is complicated by the large number of factors present and by their interdependence. Additionally, any disruption of a steroid or its receptors has implications for the normal level and function of both steroids and other signalling pathways within the ovarian system. One such example is the ArKO mouse, where the disruption of aromatase prevents oestrogen synthesis, but also causes raised androgen levels (Fisher *et al.*, 1998; Britt *et al.*, 2001).

Over the period of follicle growth and development the steroidal milieu alters in response to gonadotrophins, activins and inhibins. Under normal physiological conditions the follicular environment can initially be considered to be androgen dominant, while during later stages of follicle development the ratio of steroids is altered towards higher oestrogen.

Table 1.1:- Mouse models have been created which are lacking the steroid receptors or a key steroidogenic enzyme, aromatase. The table illustrates the key phenotypic observations which relate to female reproduction and ovarian morphology.

Knockout mouse	Gene Affected	Main ovarian phenotype	Key References
ArKO	Aromatase	No oestrogen production; elevated serum gonadotrophins; infertile due to disrupted follicle development; ovarian morphology deteriorates with age potentially due to chronic exposure to high levels of androgen and gonadotrophins in addition to the loss of oestrogen; evidence of Sertoli cell-like structures	Fisher *et al.*, 1998 Britt *et al.*, 2001
ARKO	Androgen receptor	Fertile but have a smaller number of pups per litter; longer oestrous cycle; produce fewer oocytes in response to superovulation; defective conformation of the COCs; increased apoptosis of granulosa cells;	Yeh *et al.*, 2002 Hu *et al.*, 2004
BERKO	Oestrogen receptor β	Severely reduced fertility; high LH; abnormal follicle development; Severity increasing with age • 3 wks – morphologically similar to wild type ovary • 5 months - abundant atretic follicles and few healthy late antral follicles or corpora lutea • 2 years – no healthy follicles	Krege *et al.*, 1998
ERKO	Oestrogen receptor α	Infertile; arrested follicle development at the pre-ovulatory stage with haemorrhagic cysts; raised LH levels resulting in high oestrogen & testosterone	Lubahn *et al.*, 1993
αβERKO	Both α and β oestrogen receptors	Infertile; anovulatory; Sex-reversed follicles – no oocyte but Sertoli like cells present.	Couse *et al.*, 1999 Dupont *et al.*, 2000

1.5.1 The Steroid Receptors

The steroids produced by the ovary have multiple actions; they feedback to the pituitary to determine gonadotrophin secretion as well as having paracrine and autocrine functions within the ovary. The androgen receptors expressed on GCs are developmentally regulated; in both rats and marmosets the expression of androgen receptors (ARs) is reduced as follicle development progresses, while in humans and sheep there is an up-regulation of AR expression in the preovulatory follicles (Horie *et al.*, 1992; Tetsuka *et al.*, 1995; Hillier *et al.*, 1997; Szoltys & Slomczynska, 2000). Analysis of mouse follicles found the AR was abundant in GCs of pre-antral and early antral stages but was not present in more developed follicles. However, atretic follicles maintain high levels of GC AR regardless of developmental stage (Cheng *et al.*, 2002). In both rat and mouse ovaries AR has also been localised to the oocyte, with its expression mainly seen in the cytoplasm (Szoltys *et al.*, 2003; Gill *et al.*, 2004). Recent analysis of $ARKO^{-/-}$ female mice demonstrates they have reduced fertility and produce fewer oocytes in response to superovulation than wild types (Hu *et al.*, 2004) (Table 1.1). Further analysis of the GCs during preovulatory follicle development shows there is a higher rate of apoptosis in ovaries lacking AR (Hu *et al.*, 2004).

Assessing the role of oestrogen in follicle development and atresia is complicated by the presence of the two oestrogen receptors in the mammalian ovary, ERα and ERβ. Each subtype of the ER has several splice variants and there are species-specific variations in expression pattern. In rodent follicles ERβ is localised to the GCs, found in all stages of growing follicle, with levels increasing as development

progresses (Sar & Welsch, 1999; Cheng *et al.*, 2002). ERα is only found in the theca and interstitial regions of the ovary, with none present in the GCs (Sar & Welsch, 1999; Schomberg *et al.*, 1999). The mRNA for both *ER* subtypes is expressed in the mouse oocyte (Hiroi *et al.*, 1999). The differing localisation pattern of each receptor and the distinct phenotypes of mice lacking ERβ (BERKO) and ERα (ERKO) mice, points to each receptor having a specific role within the follicle (Table 1.1).

1.5.2 Steroids and Maintenance of Ovarian Cell Phenotype

Although the reproductive tract of fetal female αβERKO$^{-/-}$ animals differentiate correctly, postnatal growth and development is severely impaired in the absence of oestrogen signalling (Couse *et al.*, 1999). The loss of oestrogen signalling results in elevated serum LH levels which may be the cause of the precocious follicle development observed in αβERKO$^{-/-}$ ovaries during prepubertal development (Couse *et al.*, 1999). In spite of the presence of primordial and growing follicles in ovaries of adult αβERKO$^{-/-}$ animals there were structures reminiscent of the male seminiferous tubules present (Couse *et al.*, 1999). Within these regions there is evidence of degenerating oocytes and differentiation of somatic cells towards the male Sertoli cell phenotype. Thus, the presence of both ERα and ERβ are required for the normal ovarian phenotype to be maintained. Potentially, the actions of oestrogen are required to ensure that oocyte and GC survival factors are transcribed (Couse *et al.*, 1999). A second model which demonstrates the importance of oestrogen for maintenance of the ovarian phenotype is the ArKO$^{-/-}$ mouse. These mice do not synthesise oestrogen although they remain responsive to it. The ovaries of these mice contain Sertoli-like cells and have raised expression of several genes

linked with male gonad development, *Sox-9*, *Sf-1* and three enzymes involved in testosterone production (Britt *et al.*, 2002; Britt *et al.*, 2004). When exogenous oestrogen was administered to the $ArKO^{-/-}$ females, there was a reduction in the Sertoli cell-like phenotype and a concurrent reduction in the genes associated with the male phenotype (Britt *et al.*, 2002; Britt *et al.*, 2004).

1.5.3 Steroids and Primordial Follicle Recruitment

There is some data which supports a role for androgen in the recruitment of primordial follicles to the growing pool, at least in the rhesus monkey. After exogenous androgen administration, ovaries of treated rhesus monkeys contained a significantly raised number of primary stage follicles, suggesting an androgen stimulated increase in primordial follicle activation (Vendola *et al.*, 1999a). The increased number of small growing follicles did not translate into higher numbers of late antral or pre-ovulatory follicles (Vendola *et al.*, 1998). The observed up-regulation of both IGF-1 and IGF-1 receptor (IGF-1R) in primordial follicles, after androgen treatment, may be the mechanism through which this steroid mediates its effect on follicle recruitment (Vendola *et al.*, 1999a, b). A stimulatory role for androgens in follicular recruitment is supported by analysis of polycystic ovarian syndrome (PCOS) in humans. Women with PCOS have a significantly correlated increase in both androgen levels and the number of growing follicles. This is consistent with the hypothesis that androgens stimulate follicle growth (Hughesdon *et al.*, 1982; Jonard *et al.*, 2003; Webber *et al.*, 2003). However, the complexities of this syndrome mean that the role of androgen in follicular recruitment can not be conclusively determined using this model. In addition to the possibility that

recruitment of primordial follicles is accelerated, the fact that PCOS ovaries could contain more follicles to start with or that the rate of follicle development is impaired are also credible.

Oestrogen may also have a role in the recruitment of primordial follicles, although its function in this process may not be consistent throughout the postnatal life of a female. The rate of recruitment of *in vitro* and *in vivo* assembled primordial follicles differs, suggesting the presence of a factor *in vivo* which limits the initial entry of follicles into the growing pool (Kezele & Skinner, 2003). Analysis of newborn rat ovaries in the presence of exogenous oestradiol allowed the effects of this steroid on primordial follicle formation and recruitment to be assessed. While the presence of oestradiol did not appear to influence the formation of primordial follicles it did inhibit the transition of primordial to primary follicles both *in vitro* and *in vivo*. However, this effect was not replicated in ovaries from older rats suggesting that oestrogens only control initial postnatal primordial follicle recruitment and that another mechanism is responsible for subsequent primordial to primary transition (Kezele & Skinner, 2003).

1.5.4 Steroids and Granulosa Cell Differentiation

Oestrogen is proposed as being involved in several mechanisms which determine follicle growth and development. Much of oestrogen's influence on the follicle occurs through its ability to augment FSH. The role of oestrogen within the ovary is complicated by the presence of two forms of the receptor, ERα and ERβ, which mediate differing responses to oestrogen. Female mice with homozygous null

mutations for either of these ERs have differing ovarian phenotypes (Table 1.1), demonstrating the differing effects of oestrogen depending on which receptor type is prevalent.

BERKO$^{-/-}$ females have a reduced response to superovulation suggesting these mice are less able to respond to gonadotrophins (Couse *et al.*, 2005). This is an interesting observation, when it is considered that a key aspect in the differentiation of the GCs is the appearance of LHRs, as these receptors are vital if the follicle is to respond to the ovulatory LH surge. The GCs of BERKO$^{-/-}$ follicles have significantly reduced levels of *LHR* mRNA (Couse *et al.*, 2005). In addition to containing abnormal levels of LHR, these GCs also have aberrant aromatase expression. Taken together these results demonstrate that signalling through ERβ is vital for normal differentiation of the GCs in the pre-ovulatory follicle (Couse *et al.*, 2005).

1.5.5 Steroids and Inter-Cell Signalling

Oestrogen also has a regulatory role in the formation and maintenance of inter-cell signalling between GCs. As previously described (section 1.3.3), the gap junctions are important for co-ordinated development of the oocyte and GCs (reviewed by Gilchrist *et al.*, 2004). They are vital in allowing the transport of signals and nutrients to the avascular regions of the follicular compartment (Eppig, 1991; reviewed by Eppig, 2001). This role becomes increasingly important with follicular growth, as the distance between GCs and the interstitial blood vessels increases. Expression of cx43, the main structural protein in gap junctions, is postulated to be regulated by oestrogen (Merk 1972; Wiesen & Midgley, 1994). Regulation of *cx43*

mRNA levels in response to oestrogen stimulation has been demonstrated in several different tissue types and species. Oestrogen has been shown to upregulate *cx43* expression in rat endometrium; the female rat preoptic area and human myometrium (Grummer *et al.*, 1994; Gulinello & Etgen, 2005; Di *et al.*, 2001). Although, these previous studies demonstrate the ability of oestrogen to upregulate *cx43* in several tissue types there is no definitive study supporting this response within the ovarian follicle (Granot & Dekel, 2002). In addition, 17β-oestradiol upregulates levels of *N-cadherin* mRNA in rodent GCs (MacCalman *et al.*, 1995; Farookhi *et al.*, 1997). The presence of the N-cadherin adhesion molecule prevents apoptosis of GCs by promoting cell-to-cell signalling (Makrigiannakis *et al.*, 1999). Thus, communication between the cells of the follicle is impaired in the absence of oestrogen. The anti-apoptotic effect of oestrogen on GCs was also confirmed in the hypophysectomised rat, with the withdrawal of oestrogen resulting in an increase in apoptosis (Billig *et al.*, 1993; Markström *et al.*, 2002).

Although oestrogen is involved in aspects of differentiation and follicular development there is evidence accumulating to suggest that the presence of oestrogen is not an absolute requirement, with follicle development and growth able to proceed with low or absent oestrogen (Coney *et al.*, 1987; Zelinksi-Wooten *et al.*, 1994; Spears *et al.*, 1998; Guo *et al.*, 2004). Evidence from knockout mice also supports the fact that follicle development can occur in the absence of normal oestrogen signalling. In the ERKO mouse follicle development only arrests at the pre-ovulatory stage demonstrating that the early stage of development can proceed in the absence of oestrogen acting via ERα (Lubahn *et al.*, 1993). The ovarian phenotype

of the BERKO mouse becomes more severe with age, the fact that at 3 weeks of age the ovaries are morphologically similar to those of wildtype mice suggests that follicle development can be supported in the absence of ERβ mediated oestrogen signalling (Krege *et al.*, 1998). The αβERKO mouse lacks both ERα and ERβ resulting in sex-reversed follicles and a complete lack of oocytes (Couse *et al.*, 1999; Dupont *et al.*, 2000). Although this demonstrates a role for oestrogen signalling in the formation or maintenance of primordial follicles the lack of normal follicular morphology does not allow any determination of the role of oestrogen on later stages of follicle growth and development. It should also be taken into consideration that these knockout mouse models have raised levels of LH as well as altered androgen and oestrogen levels (Lubahn *et al.*, 1993; Krege *et al.*, 1998). Thus, the phenotypes cannot purely be considered to be a result of lost oestrogen receptor signalling. Oestrogen is also able to act through non-classical pathways that do not involve either the α or β forms of its receptor (Moore & Evans, 1999).

1.5.6 Effects of Androgens on Follicular Growth and Atresia

In contrast to the previously described stimulatory effect on follicle recruitment, androgens have been implicated as having a role in atresia and cell death in rodents. Analysis of rat ovaries, demonstrates an apparent increase in apoptosis after administration of testosterone (Billig *et al.*, 1993). Subsequent studies on rhesus monkey and human ovaries, and mouse follicles *in vitro*, do not support an apoptotic effect of androgen (Murray *et al.*, 1998; Vendola *et al.*, 1998; Weil *et al.*, 1998 & 1999; Otala *et al.*, 2004). The conflicting results could represent a species-specific role of androgen within the ovary. However, the rat studies described above all

utilised the hypophysectomised rat as a model. Thus, the loss of gonadotrophins may explain why these studies gave conflicting results as to the effect of androgen administration. High levels of apoptotic GCs within a follicle are a sign that follicle health is poor and is the first observable sign of a follicle entering atresia. However, the presence of apoptotic cells should not automatically lead to a follicle being categorised as unhealthy. It should be remembered that apoptosis of GCs around the antral cavity is implicated in the formation of the antrum in healthy follicles (Baker *et al.*, 2001).

Although follicle development can occur in the absence of androgen, there are reports of it having a stimulatory effect on mouse follicle development *in vitro* (Spears *et al.*, 1998; Murray *et al.*, 1998) (See Table 1.2). Follicles cultured in the presence of non-aromatisable androgens have improved rates of growth and antral development. This observation can be attributed to a direct effect of the androgens, as their non-aromatisable nature prevents conversion to oestrogens. In the rhesus monkey, androgens also seem to promote follicular growth through the AR (Vendola *et al.*, 1998; Weil *et al.*, 1998 & 1999) (See Table 1.2).

Administration of androgen to an *in vivo* primate model (rhesus monkey) does not result in any increase in apoptosis; there is actually a reduction in the numbers of apoptotic GCs (Vendola *et al.*, 1998) (See Table 1.2). The administration of androgens only continued for 10 days, thus, it is possible that in the long-term androgens would result in increased rates of apoptosis. However, it would be difficult to distinguish any direct effect of androgen on increased apoptosis. The fact

that androgen recruits more follicles into the growing pool yet does not increase ovulation rate, means that there will be a higher number of follicles undergoing atresia. Culture of human ovarian cortical slices, in the presence of androgens, also adds weight to an anti-apoptotic effect of androgen. Non-aromatisable androgen, dihydrotestosterone (DHT), significantly reduces the level of apoptotic DNA in the ovary (Otala *et al.*, 2004). Thus, this effect is not mediated through conversion of androgen to oestradiol, and is a true effect of androgen rather than oestrogenic activity. In human ovaries, staining for Ki67, a marker of cell proliferation, shows that follicles of PCOS ovaries, although abnormal in appearance, are actively proliferating rather than undergoing atresia as originally assumed (Takayama *et al.*, 1996). The use of ovaries from BERKO$^{-/-}$ mice may help to clarify the role of androgen in the rodent ovary. The early stages of follicle development in these mice appear normal, although there is an increase in the recruitment of primordial follicles into the growing pool (Cheng *et al.*, 2002). However, later antral stage development is severely impaired with high numbers of follicles entering atresia. Interestingly, these BERKO$^{-/-}$ ovaries have abnormal localisation of the AR. Normally the level of AR in late antral follicles declines, but this was not found to be the case for the follicles of the BERKO$^{-/-}$ mice (Cheng *et al.*, 2002). The addition of an anti-androgen rescued the late antral follicles from atresia, suggesting that the apoptotic effects of androgen may be stage specific, having a stimulatory effect on pre- and early-antral follicles but inducing atresia by the late antral phase of development (Cheng *et al.*, 2002). Thus, there is a growing body of evidence to support the concept that androgens stimulate follicular development in several species as opposed to having an apoptotic effect.

Table 1.2:- Table showing details of androgen studies

Study	Species & Study Type	Androgens of Interest	Dose and Length of Exposure	Age of Females	Main Findings	Interpretation
Anderiesz & Trounson, 1995	Mouse *in vitro* oocyte maturation	Testosterone	10, 20, 40, 60 and 80 μM	–	Dose-dependent inhibitory effect on meiotic maturation & embryonic development (number of fertilised oocytes developing to blastocyst stage)	Testosterone has negative effect on maturation & developmental competence of immature mouse oocytes
Andersen 1993	Human Clinical Study	Testosterone Androstenedione	Assessed steroid concentrations in aspirated FF after ovarian stimulation	–	Significant increase in pregnancy rate with high oestrogen/androgen ratio in FF	Increased ratio of oestrogens/androgen is beneficial for oocyte developmental competence & higher IVF pregnancy rates
Billig et al., 1993	Rat *in vivo*	Testosterone Dissolved in ETOH	0.5mg/100μl injection Injected every 12 hrs from D24 to D28	Hypophysectomised at 22 days of age	Androgen induced reduction in ovarian weight. Increase in apoptotic DNA fragmentation in GCs (early antral & pre-antral follicles)	Androgens enhance granulosa cell apoptosis
Gill et al., 2004	Mouse *in vitro* oocyte culture	Testosterone DHT Dissolved in ETOH.	250nM for 16 hrs to assess GVBD 100, 250 & 500 nM for 16 or 7 hrs to investigate MAPK signalling	Ovaries removed from 4-5 wk old animals	250nM Testosterone reversed IBMX inhibition of GVBD Both androgens stimulated MAPK signalling in oocyte	Androgens promote mouse oocyte maturation and signalling *in vitro*
Grupen et al., 2003	Pig *in vitro*	FF steroids – Androstenedione Testosterone Endogenous FF steroids	Androstenedione – Adult 70ng/ml Pre-pubertal 16 ng/ml Molar ratios compared	Adult and pre-pubertal animals. *in vitro* oocyte maturation	Cleavage rate, blastocyst formation, trophectoderm and total cell number all greater for adult oocytes in adult FF over pre-pubertal FF	Effects on oocyte developmental competence may be due to differing steroidal environment
Lutz et al., 2001	*Xenopus* *in vitro*	Testosterone Dissolved in ETOH	Testosterone or androstenedione 1, 5, 10, 50, 100, 500 nM 16 hrs of culture	Not given	Androgens activate oocyte maturation via the classical AR signalling pathway	Androgens are the primary steroids produced by *Xenopus* ovaries & stimulate oocyte maturation

Table 1.2 continued:- Table showing details of androgen studies

Study	Species & Study Type	Androgens of Interest	Dose and Length of Exposure	Age of Females	Main Findings	Interpretation
Moor et al., 1980	Sheep *in vitro* follicle culture & oocyte transfer to oviduct	Testosterone Androstenedione	Steroid Treatment groups Oestradiol alone or Oestradiol + Testosterone (1μg/ml) + Androstenedione (1μg/ml) + 17α-progesterone & progesterone (alongside inhibitors of steroidogenesis)	Cycling females injected with PMSG on D8-10 of cycle. Ovaries recovered 36-40 hrs post-PMSG	Steroid inhibitors caused altered fertilisation rates. Addition of exogenous steroids to culture partially reversed negative effects of steroid inhibitors	Steroids play a role in the process of oocyte maturation
Murray et al., 1998	Mouse *in vitro* ovarian follicle culture	Androstenedione DHT Dissolved in ETOH	3% anti-androgen serum from D1-5 of culture OR the above serum with an excess of Androstenedione (1μg/ml)	Pre-antral follicles from ovaries of 24 day old animals	Follicles cultured in anti-androgen serum (no androgens) had significantly slower rate of growth – reversed by addition of androstenedione Low FSH impairs growth rates & antral development – addition of DHT restored to near normal	Development of ovarian follicles in culture is stimulated by the presence of androgens
Otala et al., 2004	Human *in vitro*	Testosterone DHT Dissolved in ETOH	Ovarian biopsy culture Testosterone and DHT 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L for 24 hrs GC cultures DHT 10^{-10} mol/L for 24 hrs	Ovarian biopsies from 30 women aged 18-38 years old and GCs from 8 women aged 27-36	Ovarian biopsy culture 10^{-9}mol/L testosterone reduced apoptotic DNA fragmentation DHT significantly reduced apoptosis at 10^{-7}, 10^{-8} & 10^{-10} mol/L GC culture DHT had no pro-apoptotic effect	Testosterone & DHT can suppress ovarian tissue apoptosis in culture
Silva & Knight, 2000	Cow *in vitro*	Testosterone DHT Dissolved in ETOH	Testosterone & DHT 100 nmol/L for 24 hrs of *in vitro* maturation culture	Ovaries recovered from abattoir, oocytes matured *in vitro*, IVF & embryo assessment	Both androgens significantly increased oocyte cleavage rate DHT increased proportion of oocytes reaching 8-cell stage No effect on blastocyst rate	Addition of androgens during *in vitro* maturation of bovine oocytes stimulates their maturation
Spears et al., 1998	Mouse *in vitro* oocyte culture	Androstenedione Dissolved in ETOH	1μg/ml - Androstenedione	Pre-antral follicles dissected from ovaries of 24 day old animals	Androstenedione caused a significant increase in growth & oestradiol synthesis	Androstenedione stimulates both follicle growth & oestradiol output in intact cultured mouse follicles

Table 1.2 continued:- Table showing details of androgen studies

Study	Species & Study Type	Androgens of Interest	Dose and Length of Exposure	Age of Females	Main Findings	Interpretation
Tesarik & Mendoza, 1997	Human *in vitro* oocyte culture	Androstenedione Dissolved in dimethylsulfoxide	1µM - Androstenedione	Immature human oocytes - unsuitable for ART	Androstenedione reduced frequency & amplitude of oestradiol induced Ca^{2+} oscillations (eventual loss of oscillations)	Androgens have detrimental effect on oocyte developmental competence by negating the Ca^{2+} mediated actions of oestradiol
Vendola et al., 1998	Rhesus Monkey *in vivo*	Testosterone DHT Subcutaneous pellets	Expt. set 1 Testosterone 4mg/kg per day for 3 days or 400µg/kg per day for 10 days Expt set 2 Testosterone 20µg/kg per day for 5 days or DHT 145µg/kg per day for 5 days	6-13 years of age	Increased number of:- • total follicles • primary follicle • small antral follicles • antral follicles Increased GC & theca cell proliferation No increase in GC apoptosis Dose & duration dependent effect. DHT & T effects were equal	Stimulatory effect of short-term androgen exposure on early stages of follicular growth Androgens stimulate proliferation of both granulosa and theca cells
Vendola et al., 1999a	Rhesus Monkey *in vivo*	Testosterone DHT Subcutaneous pellets	Concentrations not specified	6-13 years of age. Ovaries removed after 5 days of steroid treatment	Testosterone & DHT caused significant upregulation of IGF-1 & IGF-1R mRNA	Androgens act to up-regulate IGF-1 and IGF-1 expression in primate ovary
Vendola et al., 1999b	Rhesus Monkey *in vivo*	Testosterone DHT Subcutaneous pellets	Expt group 1 Testosterone 20µg/kg or DHT 145µg/kg for 5 days Expt group 2 Testosterone 400µg/kg for either 3 or 10 days	6-13 years of age. Ovaries removed after 3, 5 or 10 days of steroid treatment	Number of primary follicles was significantly increased Comparable effects of testosterone and DHT IGF-1 and IGF-1R mRNA significantly up-regulated in oocytes of primordial follicles	Androgens promote the initiation of follicle development perhaps via an IGF-1 mediated signalling pathway
Weil et al., 1998	Rhesus Monkey *in vivo*	Testosterone Subcutaneous pellets	Testosterone 4mg/kg for 3 days or 0.4mg/kg for 10 days	6-13 years of age. Ovaries removed after 3 or 10 days of steroid treatment	Significant changes to AR mRNA levels – increase in GCs & decrease in theca & stroma. High levels of AR associated with GC proliferation & absence of apoptosis	AR is regulated by testosterone & androgens may drive primate follicular growth

Table 1.2 continued:- Table showing details of androgen studies

Study	Species & Study Type	Androgens of Interest	Dose and Length of Exposure	Age of Females	Main Findings	Interpretation
Weil et al., 1999	Rhesus Monkey *in vivo*	Testosterone Subcutaneous pellets	4mg/kg for 3 days or 0.4mg/kg for 10 days	6-13 years of age	GC FSHR mRNA significantly increased in both testosterone treatment groups	Androgens stimulate FSHR and up-regulate androgen conversion to oestrogen
White et al., 2005	*Xenopus* & mouse *in vitro*	Androstenedione Androstenediol 19-nortestosterone Dissolved in ETOH	16 hr incubation with:- *Xenopus* Androstenedione 100 & 500 nM Androstenediol & Testosterone 1, 10, 100 & 1000 nM 19-nortestosterone 10, 100, 1000 nM Mouse Testosterone 250 & 300 nM Androstenediol 250 & 300 nM	*Xenopus* – detail not given Mouse – 4-5 wk old	*Xenopus* & Mouse Androgens increased oocyte maturation (determined by meiotic progression)	Through classical AR signalling androgens trigger meiotic progression of the ooctye in both *Xenopus* and mouse
Xia & Younglai, 2000	Human Clinical Study	Assessed Testosterone concentrations	Assessed steroid concentrations in pooled FF after ovarian stimulation. Testosterone levels of 0-5; 5.1-10 & 11-19 ng/ml	24-40 year old women undergoing ICSI treatment	No correlation of FF testosterone concentrations with:- • oocyte morphology • fertilisation rate • embryo development oestradiol:testosterone ratio associated with differences in oocyte development	oestrogen:testosterone ratio >200 associated with higher proportion of high grade oocytes recovered after ovarian stimulation
Zelinksi-Wooten et al., 1993	Rhesus Monkey *in vivo*	Aromatase inhibitor Administered orally	Aromatase inhibitor 2 doses of 1-1.25g	Not given	Elevation in circulating androstenedione/oestradiol and testosterone/oestradiol ratio No alteration in growth or number of follicles or % of atretic oocytes Oocyte nuclear maturation delayed	High androgen/oestrogen ratio impaired oocyte maturation *in vivo*

KEY

AR = Androgen Receptor
ART = Assisted Reproductive Techniques
DES = diethylstilboestrol
DHT = Dihydrotestosterone
ETOH = Ethanol
FF = Follicular Fluid
FSH(R) = Follicle Stimulating Hormone (Receptor)
GC = GCs
GVBD = germinal vesicle breakdown
PMSG = Pregnant Mares Serum Gonadotrophin

1.6 OOCYTE ACQUISITION OF DEVELOPMENTAL COMPETENCE

Developmental competence consists of a range of processes occurring in both the nuclear and cytoplasmic compartments of the oocyte. When complete these result in an oocyte which is meiotically competent (able to resume meiosis) and is capable of supporting the key post-fertilisation events including the cortical reaction; pronucleus formation; early cleavage steps; activation of the embryonic genome and progression through the pre-implantation stages of development to form a blastocyst.

The processes by which an oocyte acquires developmental competence occur during oocyte growth and during the final post-LH surge nuclear and cytoplasmic maturation stages. Although many studies investigating developmental competence only consider pre-implantation embryonic development, the condition of the oocyte can influence later development, potentially extending into adult life. The role of oocyte viability does, however, become harder to determine with increased development. The full range of molecular events involved in the acquisition of oocyte competence is as yet unknown.

In the mouse the activation of the zygotic genome occurs at the 2-cell stage, so prior to this the oocyte is itself required to support early embryonic development. Extensive studies have demonstrated that stepwise acquisition of competence occurs with oocyte growth and follicle development, particularly from the late antral stage onwards, and is influenced by the bi-directional communication between the oocyte and the somatic cells of the follicle (Eppig, 2001; Matzuk *et al.*, 2002; Murray & Spears, 2005). Developmental competence is achieved when the required

modifications have been made to both the nucleus and the cytoplasm of the oocyte in an integrated fashion (Moor *et al.*, 1998).

The organisation of the chromatin is vital for correct gene expression and developmental competence. Chromatin conformation partly determines whether the oocyte is capable of undergoing nuclear maturation. When Debey *et al.* (1993) analysed oocytes from antral follicles, the chromatin structure fell into two groups, those with a surrounded nucleolus (SN) and those with a non-surrounded nucleolus (NSN) (Figure 1.4). Stained chromatin of SN oocytes was compacted around the nucleolus, while NSN oocytes had a more diffuse staining pattern (Mattson & Albertini, 1990; Debey *et al.*, 1993; Zuccotti *et al.*, 1995). The chromatin pattern was found to predict whether the oocytes could develop past the 2-cell embryo stage after fertilisation: SN oocytes were able to develop further, while development of the NSN oocytes was blocked at the 2-cell stage (Zuccotti *et al.*, 1998). There is a significantly raised level of gene expression in SN compared to NSN oocytes which correlates to the increased developmental competence observed in SN oocytes (Christians *et al.*, 1999). This chromatin remodelling coincides with the laying down of genomic imprints on the oocyte genome, also a vital step in the acquisition of developmental competence.

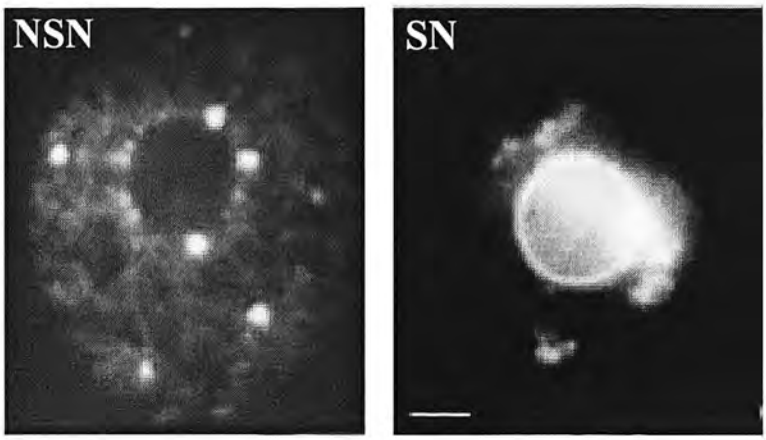


Figure 1.4:- Hoescht stained images of a non-surrounded nucleolus (NSN) GV oocyte and a surrounded nucleolus (SN) GV oocyte. These oocytes have different transcriptional activity and developmental competence. Images taken from Bouniol-Baly *et al.*, 1999.

1.6.1 Nuclear Maturation

Nuclear maturation is defined as the resumption and completion of meiosis by the oocyte in response to the LH surge (reviewed by Mehlmann, 2005). This final maturation of the oocyte occurs over a relatively short time span in contrast to the more protracted earlier stages of oocyte development. It is characterised by germinal vesicle breakdown, condensation of the oocyte chromosomes and progression through the final stages of meiosis I before arrest at metaphase of meiosis II (Eppig *et al.*, 1994) (See also Section 1.6.1.1). Being competent to complete nuclear maturation does not mean that an oocyte is developmentally competent. This is because developmental competence requires a series of nuclear and cytoplasmic processes to occur jointly. Although maturation of the nuclear and cytoplasmic compartments can proceed independently of one another integration of these processes is required if full developmental potential is to be achieved (Eppig *et al.*, 1994; Moor *et al.*, 1998).

Resumption of meiosis can occur in the absence of gonadotrophin stimulation, as oocytes removed from the antral follicular environment undergo spontaneous germinal vesicle breakdown (GVBD) and other processes associated with nuclear maturation, including chromatin condensation and resumption of meiosis (Pincus & Enzmann, 1935). Although these spontaneously matured oocytes appear morphologically normal, in some species the subsequent developmental competence is reduced. This demonstrates that, although these oocytes have undergone nuclear maturation, not all the processes required for the acquisition of developmental competence have occurred (Thibault, 1977; Niwa *et al.*, 1976; Leibfried & Bavister,

1983). However, examination of spontaneously matured mouse oocytes found that these oocytes had obtained full developmental competence (Schroeder & Eppig, 1984). The reduced developmental competence of spontaneously matured oocytes observed in early experiments could be due to inadequacies in the culture media, with successful maturation of isolated cumulus-oocyte complexes (COCs) now possible.

The observation that oocytes could spontaneously mature led to the hypothesis that there is a factor which inhibits nuclear maturation present in the follicle or alternatively that there is activation of a maturation promoting factor (MPF) (Tsafriri & Channing, 1975; Mitra & Shultz, 1996). cAMP is part of a signalling pathway involving PDE3A which has been shown to be a vital component in the maintenance of meiotic arrest (Shitsukawa *et al.*, 2001; reviewed by Mehlmann, 2005). The hypothesis that an active factor was involved in nuclear maturation led to the isolation of a meiosis activating sterol from follicular fluid (FF-MAS). This is able to overcome the negative effects of cAMP, stimulate resumption of meiosis and is positively regulated by gonadotrophins (Byskov *et al.*, 1997; Xie *et al.*, 2004).

1.6.1.1 Meiosis in the Oocyte

An oocyte within the growing follicle is held in meiotic arrest until exposed to the LH surge during pre-ovulatory follicle development. This hormonal stimulation triggers a series of events which cause the oocyte to re-enter meiosis and undergo classical oocyte maturation. The end result of these processes is production of an

oocyte arrested in metaphase of the second meiotic division which is ready for fertilisation.

One of the earliest events leading to the resumption of meiosis is the entry of cyclin B to the germinal vesicle. Immediately after this there is GVBD and MPF is activated as the oocyte enters metaphase I. The kinetics of MPF activity will act to determine the duration of the first meiotic division. At the onset of meiosis I there is activation of the microtubule organising centres (MTOCs), these sites represent regions where there is distinct polymerisation of the microtubules (Figure 1.5). There is preferential activation of those MTOCs that are located close to the chromosomes and the microtubules around these regions are stabilised. These microtubules then undergo organisation into a bipolar spindle array around the oocyte chromosomes (Figure 1.5).

Once the formation of the bipolar spindle is complete the chromosomes align at the spindle equator and form the metaphase plate (Figure 1.5). At this stage in meiosis the kinetochores of the bivalent chromosomes are not able to interact with or anchor the microtubules. This situation persists for the majority of the first meiotic metaphase. In the absence of this interaction an alternative mechanism is required to align the chromosomes on the spindle equator. It appears that this requirement is fulfilled by "polar wind" whereby the force exerted by the spindle microtubules against the arms of the chromosomes causes them to move and form the metaphase plate (Brunet & Vernos, 2001). This polar wind is mediated by microtubule motors such as Kif4 and Kif22, associated with the chromatin (Vernos *et al.*, 1995;

Bringmann *et al.*, 2004). Although the chromosomes are located at the spindle equator their accurate alignment only occurs after the kinetochores become activated and trigger the formation of K-fibres (Brunet *et al.*, 1999). The transition of metaphase to anaphase does not occur until the spindle microtubules are attached to the kinetochores and the chromosomes are properly aligned to form the metaphase plate. Premature transition into anaphase is prevented by the spindle assembly checkpoint and only occurs when the spindle has migrated from its initial location at the centre of the oocyte to the periphery. This peripheral localisation of the spindle is vital for the asymmetric division that is necessary to maintain the maternal cytoplasmic stores in the oocyte rather than them being lost in the polar body. Separation of the chromosomes represents the exit of the oocyte from the first meiotic metaphase into anaphase. Once the first meiotic spindle has reached the periphery of the oocyte and there is degradation of cyclin B there is extrusion of the first polar body (Brunet & Maro, 2005).

Immediately after the first polar body has been extruded there is formation of the second meiotic spindle and the oocyte enters the second meiotic arrest maintained by the action of Cytostatic Factor (CSF) (Brunet & Maro, 2005). The meiotic spindle remains at the periphery of the oocyte and is maintained as a stable structure in this location during the meiotic arrest. Oocytes will remain arrested in meiosis II for several hours with the chromosomes perfectly aligned on the metaphase plate and high MPF activity. It is not until fertilisation (or artificial activation) that the spindle will rotate and the extrusion of the second polar body will occur as the second meiotic division progresses (Brunet & Maro, 2005).

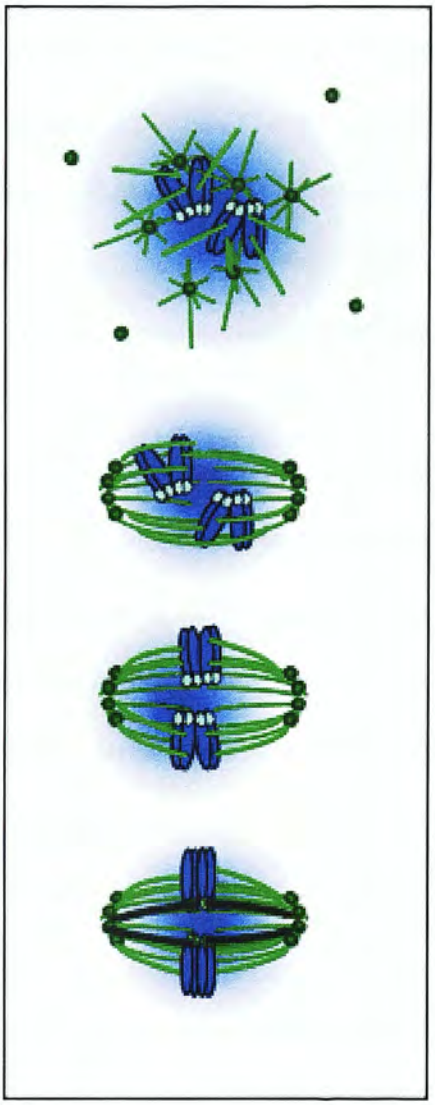


Figure 1.5:- Diagram representing formation of the meiosis I spindle in the oocyte. Activation of microtubule organising centres (MTOCs) causes polymerisation of the surrounding microtubules. There is preferential activation of those MTOCs that are located close to the chromosomes. Microtubules are organised into a bipolar spindle array around the oocyte chromosomes which then align at the spindle equator and form the metaphase plate. Accurate alignment only occurs after the kinetochores become activated and trigger the formation of K-fibres. Blue lines represent chromosomes; MTOCs are dark green; light blue discs represent kinetochores; light green represents polymerised microtubules around the chromosomes. Image is taken from Brunet & Maro, 2005.

1.6.2 Cytoplasmic Maturation

In addition to the nuclear maturation occurring after LH stimulation as described above (Section 1.6.1), the oocyte cytoplasmic compartment must have been modified during follicle development for full developmental competence to be acquired (Albertini, 2003). Within this thesis, the term "cytoplasmic maturation" is defined as encompassing all the incremental changes in the cytoplasmic compartment that lead to the step wise acquisition of competence occurring over the period of oocyte growth. The aspects of cytoplasmic maturation that are initiated in response to the LH surge are defined as final LH-induced cytoplasmic maturational processes. These changes precede and then overlap with the process of nuclear maturation. The processes of cytoplasmic maturation are very important in determining oocyte quality (Albertini, 2003). This is demonstrated by the fact that transfer of a nucleus from a developmentally incompetent oocyte to the cytoplasm of a competent oocyte results in complete developmental potential being acquired (Obata *et al.*, 1998).

One important aspect of cytoplasmic maturation is the ability to ensure that only a single sperm enters the oocyte. Polyspermy is prevented by the release of cortical granules which act to alter the ZP structure such that further sperm binding is impaired (Miller *et al.*, 1993; reviewed by Albertini, 2003). The ability to release these cortical granules is obtained during cytoplasmic maturation with immature oocytes demonstrating higher levels of polyspermy than mature oocytes (Ducibella *et al.*, 1990; Abbott *et al.*, 1999). Production of the cortical granules within the mouse oocyte has finished by the time the period of oocyte growth is complete (Ducibella *et al.*, 1988; Albertini, 2003). However, although present in the oocyte the cortical

granules are not yet competent to fuse with the membrane and trigger the cortical reaction in response to sperm entry. This final functional ability is only acquired during final LH-induced cytoplasmic maturation and actually depends upon the initiation of calcium waves triggered by fertilisation (Albertini, 2003).

One of the later aspects of competence acquired by the oocyte is the ability to generate calcium (Ca^{2+}) waves after fertilisation. The mechanisms required to initiate and maintain these Ca^{2+} waves are constructed by the time the end of the oocyte growth phase is reached. However, final LH-induced cytoplasmic maturation is required before Ca^{2+} oscillations can be successfully activated (Cheung *et al.*, 2000). In order that the oocyte can respond to sperm penetration with an appropriate release of intracellular Ca^{2+} and become activated it must be able to respond to inositol 1,4,5-trisphosphate (IP3) (Xu *et al.*, 2003). Oocytes become capable of responding to exogenous IP3 as metaphase II is reached during nuclear maturation (Mehlmann & Kline, 1994).

Cytoplasmic maturation is also required if the male pronucleus is to form correctly, with protein synthesis during development being vital to this oocyte mediated process (Albertini, 2003. In several species, for example hamster, the conversion of the sperm nucleus to a male pronucleus requires glutathione, levels of which normally rise during the final LH-induced cytoplasmic maturation. If synthesis of this factor is impaired the normal decondensation of the sperm nucleus fails to occur (Perreault *et al.*, 1988; Sutovsky & Schatten, 1997). The oocyte is also responsible for the sperm histone remodelling, the histone exchange activity necessary for this is

acquired over the period of oocyte growth and correlates to the time when meiotic competence is acquired (McClay *et al.*, 2002).

1.6.3 Oocyte Maturation

Within this thesis, the term "oocyte maturation" describes all the processes that together result in the incremental increases in developmental potential that occur over the period of oocyte growth that corresponds to the time of pre-antral and antral follicle development prior to the LH surge. This terminology encompasses the acquisition of meiotic competence as well as the cellular alterations to nuclear and cytoplasmic components that occur largely over the later growth phase of the oocyte. Thus, "oocyte maturation" is not used to cover only the specific process of nuclear maturation that occurs in response to the LH surge.

1.7 EFFECTS OF STEROIDS ON THE OOCYTE

When the steroidogenic pathways of the follicle are disrupted, by preventing the conversion of cholesterol to pregnenolone, the maturation of the ovine oocyte is affected leading to severely reduced fertilisation rates (Moor *et al.*, 1980). In this study by Moor *et al.* (1980) steroidogenesis was inhibited in cultured follicles at one of two stages: conversion of cholesterol to pregnenolone or by blocking the action of the 17α-hydroxylase enzyme system. Follicles were cultured for 24 hours with inhibition of steroidogenesis occurring for either the entire 0-24 hour culture period or 16-24hours of the culture time. The oocytes were subsequently fertilised *in vivo* before being recovered and assessed for pronuclear formation and development. In

those oocytes which had not undergone successful fertilisation the stage of meiosis (MI or MII) and whether any sperm penetration or polyspermy had occurred was recorded (Moor *et al.*, 1980). Using this ovine culture system it was found that higher fertilisation rates can be achieved by the administration of exogenous steroids, even in the absence of cholesterol cleavage (Moor *et al.*, 1980). Although it is accepted that appropriate androgen and oestrogen exposure is required for normal oocyte maturation and developmental competence, the precise mechanisms involved are not well understood. More detailed analysis of the individual effects of each of these steroids has proved difficult due to the complex feedback pathways present within the follicle. It appears that the ratio of androgens to oestrogens as well as the absolute concentrations are important to the acquisition of developmental competence (Moor *et al.*, 1980; Andersen, 1993; Zelinski-Wooten *et al.*, 1993; Xia & Younglai, 2000).

The spontaneous maturation of mammalian oocytes removed from the follicular environment has made investigation of the factors that influence maturation more complex. *Xenopus* oocytes do not undergo this spontaneous maturation process, thus more is known about the role of steroids on oocyte maturation in this species. When the maturation process of *Xenopus* and mammalian oocytes are compared there are some significant similarities. Both maintain meiotic arrest though high intracellular cAMP levels and comparable signalling mechanisms control the resumption of meiosis. In *Xenopus*, androgens have been identified as the main determinant of oocyte maturation (Lutz *et al.*, 2001). As several aspects of oocyte maturation are conserved between *Xenopus* and mammals, it is plausible that androgens may also

play a key role in mammalian oocytes (White *et al.*, 2005). However, the effect of androgens on the mammalian oocyte remains undetermined.

There are conflicting data available regarding the effects of androgen exposure on the mammalian oocyte. Androgen was largely believed to be detrimental to the acquisition of developmental competence (Anderiesz & Trounson, 1995). However, more recent studies have questioned this, and in some cases proposed a beneficial effect of androgen exposure (Murray *et al.*, 1998, 2005; Silva & Knight, 2000). In support of this, it has been observed that when physiological concentrations of androgen are added to bovine *in vitro* maturation media the cleavage rate of embryos improves (Silva & Knight, 2000). This observed improvement in embryo development was not maintained throughout pre-implantation development, with normal rates of blastocyst formation occurring (Silva & Knight, 2000). There is now an accumulation of studies, across several species, which refute the previously described negative effects of androgen on the oocyte (bovine - Silva & Knight, 2000; murine – Murray *et al.*, 1998, 2005; porcine – Grupen *et al.*, 2003). Potentially, some of these differing results are due to the wide range of androgen concentrations used. Studies which demonstrate reduced developmental competence after exposure to extremely high levels of androgen may be the result of a toxic effect at these concentrations. The timing of androgen exposure may also impact on the results, early in follicular development the follicular environment is dominated by androgens while over later stages of development oestrogen is more prevalent. Steroidal exposure which more closely mimics this normal physiological pattern of hormonal

concentrations may be expected to have a less detrimental effect on developmental competence *in vitro*.

When mouse oocytes are isolated from the follicle, they undergo spontaneous GVBD in response to the reduction in cAMP. However, it is possible to prevent this aspect of oocyte maturation by artificially maintaining cAMP levels. Using this model, exposure to androgens has been shown to illicit a stimulatory effect on oocyte maturation, resulting in GVBD and mitogen activated protein kinases (MAPK) signalling (Gill *et al.*, 2004). This result parallels observations in *Xenopus* oocytes, where androgen is thought to be the primary steroid capable of determining maturation (Lutz *et al.*, 2001). Although, this study adds weight to the argument that androgens have a positive effect on the oocyte, it may still be the case that inappropriate exposure to this steroid could cause adversely precocious maturation. In contrast, the presence of oestradiol appears to inhibit the process of nuclear maturation in several species, including pig, mouse and bovine oocytes (Li *et al.*, 2004; Beker *et al.*, 2002; Beker-van Woudenberg *et al.*, 2004). Utilisation of the $ArKO^{-/-}$ mouse demonstrates that the acquisition of developmental competence does not depend upon the presence of oestrogen (Huynh *et al.*, 2004). Thus, when these studies are considered in conjunction, it appears that although oestrogen is not required for oocyte competence, the presence of oestrogen at an inappropriate stage of development or in excess may impair oocyte maturation.

Steroidal exposure has an impact on cytoplasmic maturation processes, as demonstrated by analysis of human oocytes' Ca^{2+} release (Tesarik & Mendoza,

1995). Oocytes exposed to oestrogen initially exhibit an overall increase in cytoplasmic Ca^{2+} levels followed by detection of Ca^{2+} oscillations (Tesarik & Mendoza 1995). Subsequent exposure to androstenedione is able to influence the oestrogen induced Ca^{2+} changes. Addition of this androgen inhibits the fluctuations in Ca^{2+} level observed after oestrogen treatment alone, this effect persists even after removal of the androgen and re-exposure to oestrogens (Tesarik & Mendoza, 1997). Oestrogen exposure appears to confer a developmental advantage to the oocyte, yet the potentially beneficial changes to Ca^{2+} release are negated by androgens. This result could be construed as demonstrating a negative influence of androgens on oocyte maturation and subsequent embryo development. However, it may be that it not just the presence of steroids which can be of significance but also the order of that exposure. Additionally, the effects of steroids on cytoplasmic maturation do not appear to be conserved between species, with oestrogen having no effect on the ability of porcine oocytes to undergo cytoplasmic maturation (Dode & Graves, 2003).

1.8 FOLLICULAR ATRESIA

1.8.1 Morphology and Mechanism

Only a very small number of the follicles present in the ovary after birth will ever fully develop and ovulate a mature oocyte, with as many as 99.9% of all follicles dying through the process of atresia. The process of atresia in follicles at different developmental stages and of each cell type appears to vary. In growing follicles that become atretic, it is the GCs which appear to be the first cell type affected. The

actions of endogenous endonucleases cause DNA fragmentation prior to any morphological changes. Subsequently GCs become pyknotic, with darkly stained nuclei, as the chromatin condenses. These shrunken pyknotic GCs are removed from the follicle by neighbouring GCs, potentially through phagocytosis. This clearance of apoptotic cells is slow as the follicle is an avascular structure, until the basement membrane breaks down during atresia. All the biochemical and morphological changes seen in GCs are consistent with programmed cell death by apoptosis. As follicular degradation progresses, the oocyte, which has lost contact with the surrounding GCs, begins to lose its shape; the ZP is invaded by cells and eventually the oocyte becomes segmented (Devine *et al.*, 2000). Primordial follicles also undergo atresia but there are significant differences when compared to the observations made in atresia of growing follicles. In contrast to atretic growing follicles, the GCs of primordial follicles appear normal in their morphology. It is the oocytes of these follicles which show the first evidence of apoptosis, with cytoplasmic vacuolisation and organelles becoming unidentifiable (Devine *et al.*, 2000; apoptosis in the ovary is reviewed by Amsterdam *et al.*, 2003). As follicles increase in size there is an increase in the percentage that become atretic.

1.8.2 Control of Atresia

Whether a follicle will become atretic or continue to grow and develop depends upon both the hormones it is exposed to and the presence of the appropriate receptors allowing it to respond. The primary determinants as to whether follicles enter atresia are the gonadotrophins. Antral follicles depend upon FSH if they are to escape atresia and acquire dominance. Follicles which have reached the pre-ovulatory stage

of growth will enter atresia if they do not receive the ovulatory LH surge. The effects of gonadotrophins as survival factors are mediated through activins, inhibins and the Igf-system (Yang & Rajamahendran, 2000). Hormonal stimulation rescues the vast majority of follicles which might otherwise become atretic, suggesting that very few follicles are lost due to inherent abnormalities in either the oocyte or the follicle as a whole. In addition to the gonadotrophins there are growth factors thought to be involved in preventing the onset of apoptosis within follicles, with both EGF and FGF being shown to be capable of preventing DNA fragmentation in cultured rat GCs (Tilly *et al.*, 1992; reviewed by Markström *et al.*, 2002).

1.9 OVULATION

Ovulation can be considered in terms of an inflammatory response followed by tissue remodelling and repair stimulated by LH. In response to the LH surge the oocyte undergoes significant changes as the germinal vesicle breaks down, the chromosomes condense and there is a resumption of meiosis (reviewed by Mehlmann, 2005). Completion of the first meiotic division is marked by the expulsion of the first polar body containing half the chromosomes. The oocyte enters the second meiotic division and is arrested at the metaphase stage due to a cytostatic factor involving *mos* kinase (c-mos), until fertilization occurs; with oocytes from *c-mos*$^{-/-}$ mice undergoing parthenogenetic activation (Hashimoto *et al.*, 1994). As the oocyte undergoes these meiotic divisions there is coordinated cytoplasmic maturation which includes the loss of the cytoplasmic processes which allowed communication between the oocyte and GCs.

In addition to influencing the oocyte, the LH surge also acts on the follicle as a whole. It causes an increase in follicular fluid volume, a weakening of the matrix between GCs and an increase in blood flow to the follicle. The GCs alter their steroidogenic pathways due to the interaction of LH with its GC receptors, such that progesterone is synthesised rather than oestrogens. As follicle development progresses, there is an increase in follicular fluid resulting in a thinning of the mural GC layers. At ovulation the follicle wall ruptures, releasing the follicular fluid and the COC which is then swept into the oviduct by the fimbria. The disruption of the follicular wall is partly due to the actions of proteolytic enzymes including collagenase, plasminogen activator and gelatinase. The residual portion of the follicle within the ovary collapses to form the corpus haemorragucum, with time the blood clot is broken down and the CL forms.

1.9.1 Mechanism of LH Stimulation of the Ovulatory Response

Prior to the resumption of meiosis, the number of gap junctions between the cumulus cells and the oocyte is reduced. LH stimulates MAPK expression in the follicular somatic cells which acts to alter the phosphorylation of cx43, a vital component in gap junction maintenance (Sela-Abramovich *et al.*, 2005). The loss of communication via gap junctions means the oocyte can no longer maintain the high cAMP levels required for the maintenance of meiotic arrest and oocyte maturation is initiated. The oocyte is capable of expressing the active form of MAPK after LH stimulation but this response is only observed after several hours, when oocyte maturation is already underway. Thus, this up-regulation of MAPK in the oocyte does not cause the loss of gap junctions (Sela-Abramovich *et al.*, 2005).

LH is able to have direct effects on the follicle via its receptors but it is also able to mediate its effects through stimulation of paracrine factors. One such pathway involves up-regulation of members of the epidermal growth factor related (EGF-like) proteins, including amphiregulin, epiregulin and beta-cellulin in response to LH stimulation (Park *et al.*, 2004). Amphiregulin and epiregulin are able to cause meiotic maturation of the oocyte to an extent comparable to LH exposure, but the response is more rapid suggesting that the normal delay in maturation represents the switching on of the EGF-like gene expression (Park *et al.*, 2004). LH stimulates metalloprotease action within the follicular cells which release the membrane-bound EGF-like proteins allowing them to act as paracrine mediators of LH in the ovulatory process (Ashkenazi *et al.*, 2005). LH and all three of the EGF-like factors investigated are able to stimulate expression of several genes vital to the ovulatory response namely *hyaluron synthase-2* (*Has2*), *cyclooxygenase-2* (*cox-2*) and *TNFalpha-stimulated gene-6* (*Tsg-6*) (Park *et al.*, 2004; Ashkenazi *et al.*, 2005). Normal ovulation depends upon the cumulus cell synthesis of a mucoid extracellular matrix largely consisting of the glycosaminoglycan hyaluronan produced by *Has2* (Eppig, 1979). The cumulus cell matrix also contains the hyaluronan binding protein Tsg-6 which is a target for prostaglandins (PGs). Cox-2 is the rate limiting enzyme responsible for synthesis in the ovary, with *cox-2* deficient females having impaired cumulus expansion and reduced ovulation rate (Lim *et al.*, 1997; reviewed by Eppig, 2001).

1.10 FERTILISATION AND PRE-IMPLANTATION DEVELOPMENT

Fertilisation occurs in the ampulla region of the oviduct when the oocyte is fertilised by a single sperm, followed by formation of pro-nuclei and the initiation of embryo development (Figure 1.6). The oocyte and cumulus cells secrete a factor which aids fertilisation due to a chemotaxic effect on sperm (Oliveira *et al.*, 1999; Sliwa, 2001). Although follicular fluid of both humans and mice has been shown to have a chemotaxic effect on sperm it is unlikely that the secretion of this factor would be restricted to the pre-ovulatory COC as only a small volume of follicular fluid enters the fallopian tube at ovulation and the fluid would not set up a reliable concentration gradient to influence the sperm. This suggests that any chemotactic factor would continue to be produced by the COC after ovulation (Giojalis & Rovasio, 1998; Oliveira *et al.*, 1999).

When a sperm binds to an oocyte, there is localised proteolytic degradation of the ZP. This enables the sperm to reach the surface membrane of the oocyte where the head of the sperm will be enveloped by microvilli. The subsequent fusion between sperm and oocyte membranes results in the male nucleus moving into the oocyte cytoplasm. This initiates a Ca^{2+} wave from the point of sperm entry due to an increase in intracellular Ca^{2+} (Saunders *et al.*, 2002; Knott *et al.*, 2003). These Ca2+ oscillations associated with oocyte activation are regulated by the sperm-specific factor, phospholipase C-zeta (PLC-zeta) (Saunders *et al.*, 2002; Kouchi *et al.*, 2004; Nomikos *et al.*, 2005). Once these stages have been successfully completed, the oocyte containing the sperm nucleus is termed a zygote. It is vital that the oocyte prevents the entry of more than one sperm, thus the zona reaction occurs to stop

polyspermy. This multi-step process prevents further sperm binding to the ZP, inhibits proteolytic breakdown of the ZP and alters the oocyte cytoplasm so no further sperm can enter. At fertilisation, the oocyte re-enters meiosis as a result of MPF breakdown by a Ca^{2+} stimulated enzyme. Completion of the second meiotic division is marked by the Ca^{2+} and protein kinase-C-mediated formation of the second polar body. The maternal and paternal chromosomes maintain their separate identities as they become surrounded by membranes to form two pronuclei (Figure 1.6). These move to a more central position and synthesise DNA in anticipation of the initiation of mitosis.

Once the pronuclear membranes break down and the chromosomes take up their positions on the mitotic spindle the zygote has achieved syngamy, the mixing of chromosomes from the two gametes. The cell undergoes mitotic division resulting in the formation of a 2-cell embryo (Figure 1.6). Embryos become transcriptionally active at a species specific stage of development; in mice this ability to synthesise its own mRNA rather than translating maternal mRNA stores starts at the 2-cell stage. Even after transcriptional activation proteins previously translated from the maternal mRNA will persist through the early stages of development resulting in a prolonged maternal effect on the embryo. The embryo continues to divide and will undergo compaction to form a morula at the 8 to 16 cell stage depending on the species, followed by development of the blastocoel cavity to form a blastocyst (Figure 1.6). Blastocysts consist of two populations of cell, the outer layer of trophectoderm and the inner cell mass (ICM).

Figure 1.6:- Pre-implantation embryo development:- After fertilisation the zygote forms the male and female pronuclei. When these combine syngamy has been achieved and the embryo undergoes mitotic cleavage. Embryo development to the hatching blastocyst stage can be supported in vitro.

The trophectoderm does not contribute to the fetus but gives rise to the trophoblast cells which are vital for implantation and placentation (reviewed by Johnson & McConnell, 2005; Armant, 2005). The fetus and much of the extra-embryonic tissue arise from the ICM (reviewed by Armant, 2005). The embryo remains surrounded by the ZP until the hatching of the blastocyst, the ZP acting to hold the blastomeres together prior to compaction. This prevents twinning and the mixing of blastomeres from different embryos which would result in the formation of a chimaera. The embryo enters the uterus between the morula and blastocyst stages: it is not until the blastocyst has hatched from the ZP that it is able to interact with the lining of the uterus allowing implantation and placenta formation to occur. Providing a fetus is healthy it will continue to grow and develop throughout gestation, which in the mouse lasts for around 19-21 days.

1.11 DNA METHYLATION IN THE MAMMALIAN GENOME

1.11.1 Regions of the Genome Containing DNA Methylation

It is perhaps surprising that the vast majority of studies that have investigated the DNA methylation patterns and dynamics of the germ cells have concentrated on the small number of imprinted genes and the phenomenon of genomic imprinting (Marchal *et al.*, 2004). These investigations have utilised and developed a range of techniques for assessing the location, pattern and quantity of DNA methylation present in the genome (Table 1.3). Although important for normal development and gene transcription the DNA methylation present at the imprinted gene loci only accounts for a small proportion (<1%) of the methylated cytosine residues that are

found in the mammalian genome. The largest proportion of DNA methylation is found in the CpG rich repeat sequences of the mouse genome (Lees-Murdock *et al.*, 2003; Rollins *et al*, 2006). This imbalance in the studies carried out means that less is known about the DNA methylation that is found in these non-imprinted genome regions and the effects that result from changes to global methylation levels at these repeat loci in different developmental contexts.

There are different types of repetitive sequences present in the genome, these include the minor and major satellite sequences; short interspersed nuclear elements (SINEs); long interspersed nuclear elements (LINEs), Intracisternal A-particles (IAPs) and endogenous retroviruses (Lees-Murdock *et al.*, 2003; Marchal *et al.*, 2004). Minor & Major Satellite Sequences are located in the juxtacentromeric regions of chromosomes and consist of repeat sequences of a few base pairs (20- to 200-bp repeats) (Lees-Murdock *et al.*, 2003; Marchal *et al.*, 2004). Although these satellite sequences are not transcribed they are thought to play a role in both centromere function and kinetochore assembly (Lees-Murdock *et al.*, 2003). The remaining repeat sequence classes are found to be interspersed across the genome at various loci. SINEs, LINEs and IAPs are all derived from transposable elements (Marchal *et al.*, 2004). These transposable elements and the endogenous retroviruses are able to undergo reverse transcription and insert into new locations in the genome (Lees-Murdock *et al.*, 2003). Further analysis of the localisation pattern of these repeat sequences demonstrates that SINEs are largely found in R-bands, LINEs are located in G-bands while IAPs are found to be widely dispersed across the full length of the chromosomes (Marchal *et al.*, 2004).

Table 1.3:- Table summarising the common techniques used to assess DNA methylation

Chemical Methods				
Method	**Application**	**Overview**	**Pros & Cons**	**Reference**
Genomic Sequencing by hybridisation	Methylation profiling of genomic DNA	Cytosine can be discriminated from 5-methylcytosine by the differing sensitivity to hydrazinolysis. Combines chemical sequencing and Southern hybridisation. Involves the chemical modification of DNA then separated on a polyacrylamide gel. Appropriate DNA sequences are then used on the gels as a probe in hybridisation experiments. The absence of bands at corresponding C positions demonstrates the presence of 5-methylcytosine.	**Advantages** Allow methylation profiling of genomic DNA **Disadvantages** Technically demanding Very low sensitivity such that large levels of radioactivity are required if high enough signals are to be obtained. Negative display for 5-methylcytosine results	Church & Gilbert, 1984
Genomic Sequencing by Ligation-mediated PCR (LM-PCR)	Used to detect 5-methylcytosine in a specific sequence of a genome. Can be used on very low levels of DNA as involves PCR amplification.	Similar to the above technique but PCR is used to amplify very small starting amounts of DNA. Chemical degradation and strand cleavage; elongation using a strand-specific genomic primer; ligation of an adaptor and elongation; PCR and visualisation. A methylated cytosine site is not cleaved so there will be no PCR fragment present.	**Advantages** Allow methylation profiling of genomic DNA **Disadvantages** Technically demanding Negative display for 5-methylcytosine results	Mueller & Wold, 1989 Pfeifer *et al.*, 1989
Bisulfite Modification	**Several methods based on this chemistry have been developed (See over the page in this table)**	Treatment of DNA with sodium bisulfite results in conversion of cytosine residues to uracil. The 5-methylcytosine is converted to uracil with much less efficiency than unmethylated cytosines. Subsequent PCR results in conversion of the unmethylated cytosine residues to thymine while 5-methylcytosine remains as cytosine.	Generally methods utilising the bisulfite chemistry provide the advantage that virtually any CpG present in the genome can be detected. The key problem with techniques using the bisulfite method is that it is particularly prone to reaction artifacts which can affect reliability (especially incomplete conversion)	Dahl & Guldberg, 2003

Table 1.3 continued:- Table summarising the common techniques used to assess DNA

Method	Application	Overview	Pros & Cons	Reference
Bisulfite genomic sequencing	Allows the methylation pattern between alleles to be distinguished Allows the methylation pattern between cell population to be distinguished	Analysis of the nucleotide sequence of the above PCR products allows the methylation status of the cytosines in the original genomic DNA to be established	**Advantages** Clear identification as to whether the original cytosine was methylated or not Robust, sensitive and reproducible Careful optimisation can be used to eliminate many of the technical problems Simple to interpret results **Disadvantages** After modification the DNA strands are no longer complementary – thus the PCR conditions may vary depending on the template strand A heterogeneous methylation status in the region of interest can influence the efficiency with which different fragments are amplified in PCR Long regions of DNA can undergo sufficient depurination such that amplification is impaired.	Olek *et al.*, 1996 Dahl & Guldberg, 2003
Methylation Specific PCR (MS-PCR)	Detects presence/absence of methylated alleles.	Requires two primer sets – one for the methylated sequence and one for the unmethylated sequence. Results determined by the presence/absence of PCR products.	**Advantages** Simple Sensitive so allows assessment of cytosine methylation if amount of tissue is limited. **Disadvantages** Specificity is restricted to the primer-binding sequences Primer design is critical and complex	Fraga & Esteller, 2002
Methylation-Sensitive single nucleotide primer extension (MS-SNuPE)	Allows a quantitative analysis of the ratio of cytosine to 5-methylcytosine.	Different nucleotide triphosphates are incorporated into the target position when bisulfite treated DNA is used as the template.	**Advantages** Good for quantitative analysis Avoids use of restriction enzymes Allows analysis of small amounts of DNA Simultaneous assessment of multiple CpG sites possible **Disadvantages** Specific primers may be difficult to design if the region of interest is in a CpG-rich area. Incomplete conversion (associated with bisulfite method) PCR bias	Dahl & Guldberg, 2003
Combined bisulfite restriction analysis (COBRA)	Estimate of methylation status	Unmethylated Taq1 restriction sites are converted to a TTGA sequence in the PCR product of bisulfite treated DNA and cannot be digested by the Taq1 enzyme.	**Advantages** Convenient Allows assessment of DNA methylation levels at specific loci even from small amounts of DNA Not as prone to false positives as some other techniques **Disadvantages** Restricted to assessing methylation at specific restriction sites. The methylation status of the restriction site may not represent that of the entire region of interest. Again technique can be limited by incomplete bisulpfite conversion	Oakeley, 1999 Dahl & Guldberg, 2003

Table 1.3 continued:- Table summarising the common techniques used to assess DNA

Enzymatic Methods				
Method	**Application**	**Overview**	**Pros & Cons**	**Reference**
Methylation Sensitive Restriction Endonucleases (MSRE)	Evaluation of the methylation status of specific DNA sequences	Restriction endonucleases are used to assess the methylation status of the recognition site. If the site is methylated then it is resistant to cleavage. The resulting fragments can be detected by either southern hybridisation or PCR.	**Advantages** Simplicity of the technique **Disadvantages** Assumes that the methylation status of the restriction site is representative of the entire CpG island or genomic region which does not necessarily hold true. Incomplete digestion of the DNA will result in an overestimate of the DNA methylation level. Can be a particular problem when high molecular weight DNA is digested (this can be compounded when the particularly sensitive technique of PCR is used for analysis). The fact that non-methylated alleles are represented by the lack of signal after PCR (i.e. negative display of results). In some cases fragments can be too small to detect using Southern hybridisation.	Havliš & Trbušek, 2002 Dahl & Guldberg, 2003
Restriction Landmark Genomic Scanning (RLGS)	Genome wide analysis of methylation status of restriction sites that are rare within the genome The restriction endonuclease NotI is often used to identify imprinted genes and CpG islands that have become methylated in human cancers.	Utilises restriction endonucleases that can only cleave their rare recognition sites if they are unmethylated. These fragments are then labelled with a radioisotope and digested using a second restriction endonuclease before being separated using gel electrophoresis.	**Advantages** Simplicity of the technique Allows genome wide searches for differentially methylated restriction sites **Disadvantages** Using RLGS the methylated alleles are negatively displayed.	Hatada *et al.*, 1991 Hatada *et al.*, 1993 Costello *et al.*, 2000
Other techniques				
5-methylcytosine Antibody	Powerful tool to study global DNA methylation	Genomic DNA is denatured and then incubated with anti-5-methylcytosine antibody. The intensity of staining, as determined by fluorescein-conjugated secondary, is proportional to the degree of methylation. The strongest signals are emitted from chromosomal regions that have particularly high density of 5-methylcytosine e.g. juxtacentromeric regions.	**Advantages** Quantitative Simple technique Suitable for assessment of global methylation Allows chromosomal DNA methylation patterns to be mapped **Disadvantages** No detail on methylation status of individual CpGs Prolonged exposure to HCl to denature the DNA can result in purinisation of the CpG dinucleotide resulting in an artificially lowered level.	Bernardino *et al.*, 2000 Dahl & Guldberg, 2003

Investigations into genomic imprinting have uncovered the important role of DNA methylation in transcriptional repression. This function is paralleled in the non-imprinted repeat sequences where DNA methylation has a role in maintaining the retroviruses and other elements in an inactive state, termed the genome defence model (Bestor, 1999; Bird, 2002; Lees-Murdock *et al.*, 2003). This role of DNA methylation is demonstrated by the analysis of endogenous retroviruses. These sequences are flanked by long terminal repeats (LTRs) which contain the enhancer and promoter elements for the retroviruses. When the DNA methylation on the LTRs is reduced there is an increase in the transcription of the retroviruses demonstrating that methylation of these regions acts to repress transcription (Lees-Murdock *et al.*, 2003). As well as transcriptional repression the presence of DNA methylation is also correlated with genomic stability. The consequence of loss of DNA methylation at classical satellite 2 and 3 sequences in humans with ICF is aberrant chromosome formation (Lees-Murdock *et al.*, 2003). An alternative theory as to the importance of DNA methylation of the repeat sequences in the genome has been proposed. This suggests that the transcription of such large amounts of DNA that do not code for functional gene products would create a large amount of "transcriptional noise" in the cells such that it could interfere with vital gene expression and the normal processes of the cell (Bird, 2002).

1.11.2 What is Genomic Imprinting?

Genomic imprinting is the parent-of-origin, allele-specific, gene expression and is determined by epigenetic modification of genes, such that gene transcription is altered while the actual gene sequence remains unchanged. Genomic imprinting results in only one inherited copy of the relevant imprinted gene being expressed in

an embryo. For paternally imprinted genes, the paternal allele is epigenetically modified to prevent transcription, ensuring that the embryo has only mono-allelic expression from the maternally inherited copy. The opposite is true of maternally imprinted genes, when only the copy inherited from the father is expressed. The fact that expression is limited to one allele means that the level of mRNA transcribed and the subsequent amount of translated protein are lower than those from genes with biallelic expression. In this way genomic imprinting acts as a dosage control mechanism (Allegrucci *et al.*, 2005). The fact that particular genes are differentially expressed, according to their parent-of-origin, means that during development the parental genomes are functionally non-equivalent (Surani 1998). Genomic imprinting is vital for normal gene expression patterns in an individual, with errors sometimes resulting in inappropriate gene transcription or repression. Within the mouse genome, approximately 80 imprinted genes have so far been identified (Beechey *et al.*, 2005). It is likely that there will be a similar number of imprinted genes in humans, although fewer have been found to date. Except where stated, this review refers to work on the mouse, as this species has been by far the most extensively studied species.

Within the mammalian genome, the majority of cytosine residues found as CpG dinucleotides (i.e. those cytosines positioned next to a guanine residue) have a methyl group added to their carbon 5-position (Costello & Plass 2001) (Figure 1.7). This equates to ~3 x 10^7 methylated cytosine residues in total (Bestor, 2000). It is this addition of the methyl group, referred to as DNA methylation, which is the best studied mechanism involved in imprinting. However, it is not the sole epigenetic

mechanism with a role in controlling imprinted gene expression. Chromatin configuration, modifications to histone proteins (phosphorylation, acetylation and methylation) and the presence on DNA of regulatory protein complexes are also all mechanisms involved in transcriptional control of imprinted genes (Meehan, 2003; Allegrucci *et al.*, 2005). In addition, there is emerging evidence that regulation of imprinted gene expression in mouse placental is dependent on histone modifications independently of DNA methylation. Several paternally repressed genes lack parent-specific DNA methylation, and genetic ablation of methylation does not lead to loss of their imprinting in the trophoblast. Instead the silent paternal alleles are marked in the trophoblast by the repressive histone modifications (dimethylation at Lys9 of histone H3 and trimethylation at Lys27 of histone H3), which may be dependent on the Eed-Ezh2 Polycomb complexes (Lewis *et al*, 2004). Studies on embryonic stem cells and early embryos suggest that this chromatin based repression is established early in development, but in the embryo, this imprinting is stably maintained only at genes that also have promoter DNA methylation (Umlauf *et al*, 2004). It is important to point out, however, that the majority of methylated DNA in the genome is not concerned with genomic imprinting. Heavy methylation of DNA is correlated to a more condensed structure which is resistant to transcription. It remains unclear as to whether the alterations to DNA methylation, chromatin configuration or histone modifications are the initial epigenetic signal causing gene repression. It may be the case that there is a system whereby several of these epigenetic modifications act co-operatively to initiate, maintain and reinforce the control of gene transcription (Nakao, 2001; Geiman & Robertson, 2002; Fuks, 2005). Thus, if an animal inherits a methylated copy of a gene from its mother and a non-methylated copy from its

father, the maternal copy will have its transcription repressed leaving the paternal copy as the only active gene. DNA methylation is an epigenetic modification which can be inherited in a stable manner. When DNA replicates the two resultant daughter strands of DNA are each hemimethylated. Stable inheritance is ensured by the action of a maintenance DNA methyltransferase which preferentially methylates hemimethylated DNA to produce properly symmetrical methylation of the DNA strands (Riggs & Xiong, 2004). Dnmt1 preferentially binds to hemimethylated DNA *in vitro* and, unlike Dnmt3a and Dnmt3b, is localised to the replication foci of dividing cells making it a strong candidate for fulfilling this role *in vivo* (Bestor 2000; Margot *et al.*, 2001). The epigenetic mark of DNA methylation is also reversible allowing gender specific patterns to be initiated in germ cells.

1.11.3 Why Did Genomic Imprinting Evolve?

The most commonly proposed theory explaining the development of genomic imprinting is the Genetic Conflict or parental investment theory (Moore & Haig 1991). This theory arose from the observation that many imprinted genes are implicated in the growth and development of the mammalian fetus or placenta. In addition to this, imprinted genes have been shown to exhibit directionality in their actions, that is the majority of the paternally expressed genes, such as *Igf2* and *Peg3*, promote fetal growth and nutrient uptake while in contrast the maternally expressed genes, such as *Igf2r* and *Gnas*, tend to curb fetal growth (Reik & Dean 2001; Tycko & Morison, 2002). Females who could restrict fetal growth and produce more offspring from their limited resources would, in the long term, be more successful. In contrast, males would derive long-term benefit from their progeny being larger

and stronger even if they achieved this to the detriment of the mother by utilising more maternal nutrients.

An alternative theory to account for the presence of imprinted genes within the genome is the Evolvability Model (Beaudet & Jiang 2002). This predicts that species which have genomic imprinting are more able to evolve in response to environmental pressures, as they can induce rapid changes by altering which of the two alleles is silenced and which is expressed. An individual organism can carry an allele which promotes growth that while imprinted has no phenotypic effects. Thus, if increased growth becomes advantageous, the relevant allele is already present in the gene pool: by rapid reversal of the imprinting, the allele can be expressed. There is also the "ovarian time bomb" concept which proposes that genomic imprinting evolved to protect the female from ovarian disease: Varmuza & Mann (1994) hypothesised that imprinting could limit the level of growth and development of any parthenogenetic embryos within the ovary, thereby preventing malignant trophoblast formation.

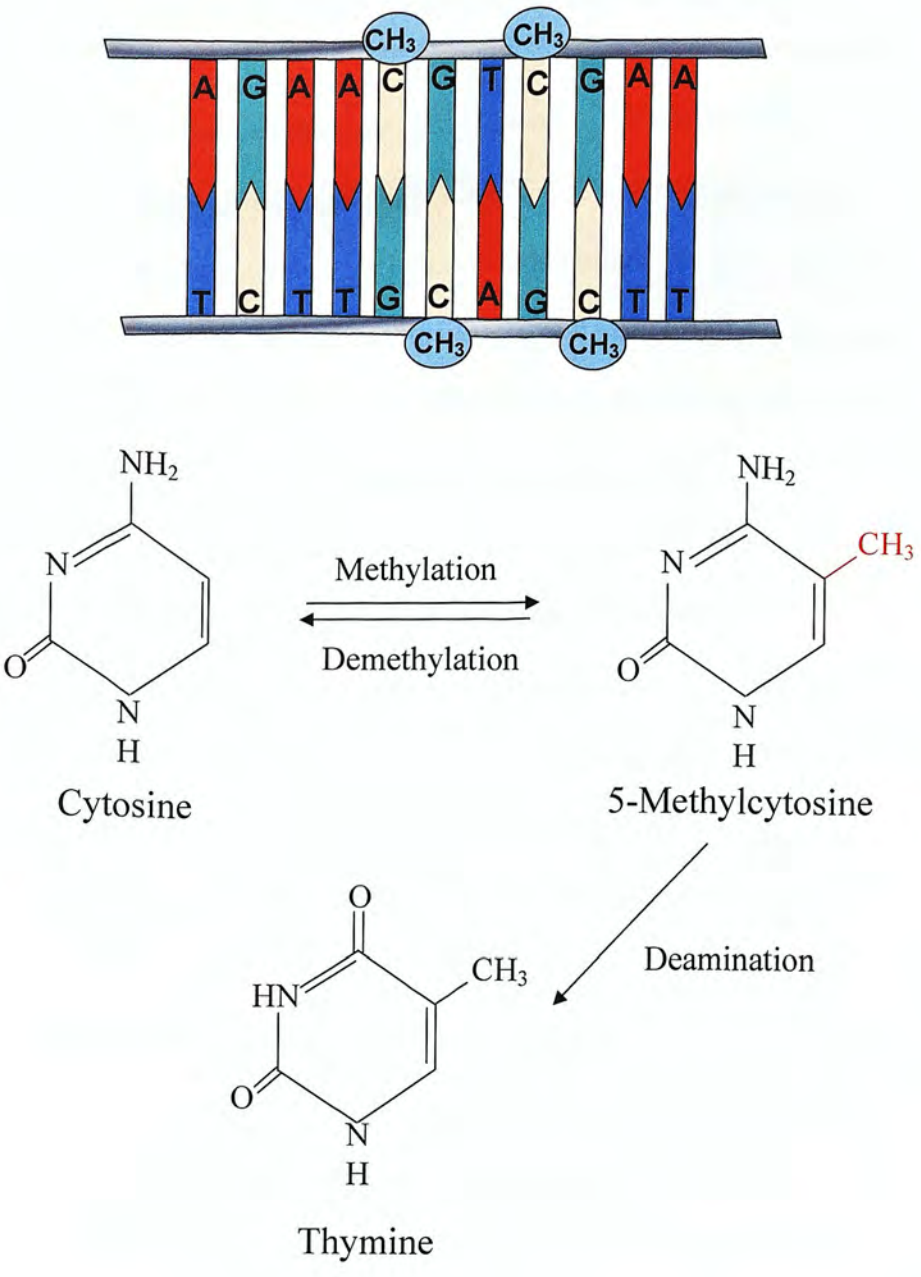


Figure 1.7:- DNA methylation involves the addition of a methyl group (CH_3) to the cytosine residue in CpG dinucleotides resulting in the formation of 5-methylcytosine. This methylation of cytosine is a reversible process and 5-methylcytosine is susceptible to deamination resulting in its mutation to thymine, unless repaired this can lead to single base mutations. A – Adenine; C – Cytosine; G – Guanine; T – Thymine.

1.12 HOW ARE DNA METHYLATION PATTERNS REGULATED?

DNA methyltransferases (Dnmts) carry out methylation of DNA; these can be broadly divided into Dnmt1, Dnmt2 and Dnmt3 families. The three families of Dnmts are related, albeit distantly, and are believed to have diverged from their common ancestors prior to the separation of the animal and plant kingdoms (Howell *et al.* 2001). Members of both the Dnmt1 and Dnmt3 families have been identified as having active transmethylase activity and their functions have been partially elucidated, with the Dnmt3 family being primarily concerned with laying down new methylation patterns while the Dnmt1 family appears mainly involved in maintenance of these patterns during cell division. Initial studies on Dnmt2 did not find any active methylation function for this protein but more recent research has challenged this concept, with work by several groups finding that this enzyme can act as a methyltransferase which targets a very specific DNA sequence, explaining the low level of identifiable activity (Liu *et al.* 2003; Hermann *et al.* 2003; Tang *et al.* 2003). Although capable of binding to methylated DNA the definitive binding specificity of Dnmt2 has yet to be determined (Hermann *et al.*, 2003). Golding & Westhusin (2003) have shown that *DNMT2* is actually the most prevalent *DNMT* in the bovine adult ovary and testis. The situation is different in *Drosophila*, with the Dnmt2 ortholog, dDnmt2, being the only Dnmt which methylates the *Drosophila* genome (Hung *et al.*, 1999, Tweedie *et al.*, 1999). More recently a role of dDnmt2 in determining the lifespan of *Drosophila* has been proposed. Over-expression of the protein allows flies to live longer, and a threshold level of dDnmt2 expression appears to be vital for normal lifespan (Lin *et al.*, 2005). Although no such

investigation has been carried out in mammals, this work in *Drosophila* introduces a possible new and intriguing role for the *Dnmt2* gene in other species.

1.12.1 Laying Down of Methylation Patterns

To allow reprogramming of the germ cell, the genome must undergo demethylation (as described later) (Hajkova *et al.*, 2002). Once the initial imprints have been removed the appropriate new pattern must be established, thus ensuring that the paternal- and maternal-specific imprints are laid down in the sperm and oocyte respectively. The enzymes which are capable of laying down the new methyl groups onto previously unmethylated DNA are from the Dnmt3 family. Members of this family which have active transmethylase activity are Dnmt3a and Dnmt3b, which share a high degree of sequence homology but have been shown to have different expression patterns and timing through development (La Salle *et al.*, 2004; Lucifero *et al.*, 2004). The third member of this family, Dnmt3l, shares sequence homology with the other enzymes but is missing the catalytic domain needed to add methyl groups onto DNA (Aapola *et al.*, 2001). After both examining the localisation of this protein and using mice with a disrupted *Dnmt3l* gene, a role in the establishment of maternal imprints in the oocyte has been hypothesised for this enzyme, as discussed below (Bourc'his *et al.* 2001; Hata *et al.* 2002)

1.12.2 Maintenance of Methylation

When a methyl group is added onto already hemi-methylated DNA during cell replication (necessary if the daughter cells are to maintain the methylation pattern of the cell undergoing mitosis), the process is termed maintenance methylation (Riggs

& Xiong, 2004. Dnmt1 has the primary responsibility for maintaining the methylation status of DNA (Bestor, 2000; Margot *et al.*, 2001; reviewed by Robertson, 2002). The most common form of this methyltransferase is that found in all somatic cells, Dnmt1s, and has been shown to be vital for development (Li *et al.*, 1992; Biniszkiewicz *et al.*, 2002). In addition there are two splice variants identified which are specific to the germ cells and early embryo (Mertineit *et al.*, 1998). *Dnmt1p* is found in pachytene spermatocytes whilst *Dnmt1o* is only identifiable in the oocyte and pre-implantation embryo (Mertineit *et al.*, 1998). It is not until embryonic day 7 (E7) that the embryo is capable of producing full length Dnmt1s protein (Mertineit *et al.*, 1998).

Interestingly, although Dnmt1 has been identified as the main maintenance methylase *in vivo*, studies *in vitro* have shown that this enzyme has a higher de novo methylase activity than either Dnmt3a or Dnmt3b (Yoder *et al.*, 1997). *In vivo* Dnmt1 de novo methylase activity has yet to be found, but the possible implications of this *in vitro* activity should be borne in mind (Howell *et al.* 2001). There are interactions between the Dnmt enzymes which enable them to carry out the functions of maintenance and de novo methylation (Fatemi *et al.*, 2002). Thus, it is difficult to classify a particular Dnmt as an exclusive maintenance or de novo methylase. Cooperation between Dnmt3a and Dnmt1 in the de novo methylation of DNA has been demonstrated (Fatemi *et al.*, 2002). Using an *in vitro* model the level of de novo methylation activity was considerably higher when both Dnmt3a and Dnmt1 were present in combination rather than individually (Fatemi *et al.*, 2002). Further assessment demonstrated that a physical interaction between these two Dnmts was

not an absolute requirement (Fatemi *et al.*, 2002). Taking these results into account it is proposed that Dnmt3a acts to add de novo methylation in a distributive fashion. This hemimethylation then attracts Dnmt1 which is able to methylate the unmodified strand at this site. In addition this pre-existing methylation seems to stimulate the ability of Dnmt1 for de novo methylation (Fatemi *et al.*, 2002; Liang *et al.* 2002). This then results in the entire DNA domain undergoing methylation in a processive manner (Fatemi *et al.*, 2002; Kim *et al.*, 2002).

1.12.3 How Does Methylation Lead to Repressed Gene Transcription?

There are several main mechanisms by which the methylation of DNA can prevent the transcription of genes (as reviewed by Attwood *et al.*, 2002). The first of these is by the methyl group causing direct interference preventing particular transcription factors from binding to methylated DNA (Iguchi-Ariga and Schaffner 1989; Lande-Diner & Cedar, 2005). The second mechanism results from methyl binding domain proteins (Mbds) binding to methylated DNA (Wade, 2001; Lande-Diner & Cedar, 2005; Figure 1.8). Of the Mbds identified to date, Mbds 1-3 and MeCP2 are involved in transcriptional repression (Nan *et al.* 1997; Fujita *et al.* 1999; Ng *et al.* 1999; Wade, 2001), while Mbd4 is thought to have a role as a mismatch repair protein (Hendrich *et al.* 1999; Hendrich & Tweedie, 2003). Mbd1 and MeCP2 both contain transcriptional repression domains which act via histone deacetylases (HDACs) (Hendrich & Tweedie, 2003). HDACs cause local deacetylation of the histone tails which in turn results in remodelling of the chromatin into a more condensed structure that is resistant to transcription (Taunton *et al.* 1996; Wade 2001). Mbd1 mediates transcriptional repression through recruitment of a histone

methylase capable of binding HDACs (Ng *et al.* 2000; Wade, 2001; Fujita *et al.* 2003), while MeCP2 acts to bind a co-repressor complex containing an HDAC (Jones *et al.* 1998; Nan *et al.* 1998), although MeCP2 has also been shown to cause transcription repression in the absence of HDAC activity perhaps through histone methylation (Nan *et al.* 1998; Yu *et al.* 2000; Wade, 2001; Fuks *et al.*, 2003). Mbd2 and Mbd3 are both components of a large protein complex, MeCP1 (Feng & Zhang 2001). MeCP1 binds methylated DNA in a non-sequence-specific manner. The binding of MeCP1 to methylated DNA is due to the presence of Mbd2 in the complex (Ng *et al.* 1999). Interestingly, the mammalian form of Mbd3 appears not to bind directly to methylated DNA (Hendrich & Bird 1998; Fraga *et al.*, 2003). The MeCP1 complex binds methylated DNA less tightly than MeCP2, which suggests that long-term transcriptional repression may be maintained by the permanent binding of MeCP2, with more transient transcriptional silencing determined by the binding of the MeCP1 complex (Ng *et al.* 1999). Although not involved in gene repression the importance of Mbd4 becomes clear when a characteristic of methylated CpG dinucleotides is considered. Where DNA is methylated, the 5-methylcytosine residue is prone to mutation through deamination to thymine (Hendrich & Tweedie, 2003; Figure 1.7). This means it is particularly important to have a repair mechanism capable of targeting this mutation (Bird, 1980; Hendrich & Tweedie, 2003). Mbd4 preferentially binds to methylated regions of the genome and has been shown to remove this mismatched thymine residue (Hendrich *et al.*, 1999; Hendrich & Tweedie, 2003). In addition, mice with mutated *Mbd4* alleles have a higher incidence of mutations than controls (Millar *et al.*, 2002; Hendrich & Tweedie, 2003).

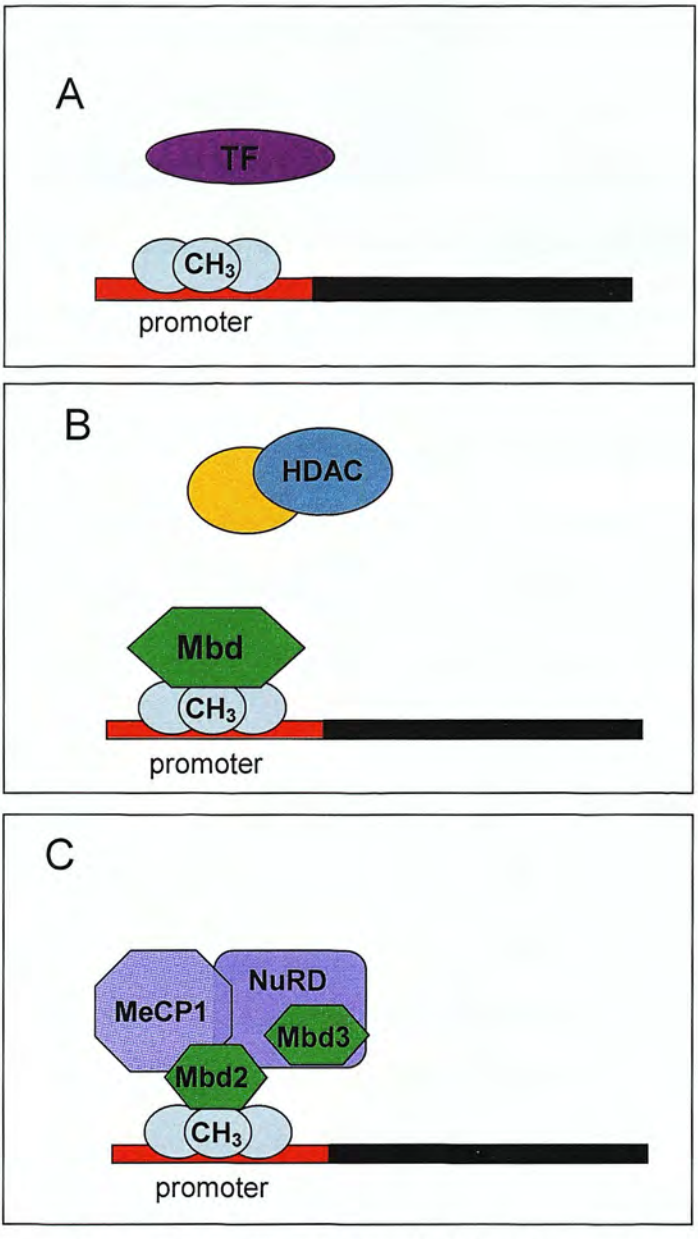


Figure 1.8:- Mechanisms which mediate DNA methylation dependent repression of gene transcription. **A** – methyl group acts as a physical barrier to prevent transcription factors binding to gene promoter. **B** – Methyl binding domain protein binds to methyl groups on DNA and then recruits co-repressor complexes containing histone deacetylases. **C** – Components of the MeCP1 activity include Mbd2 and the NuRD complex containing Mbd3. Mbd2 binds to methyl groups on the DNA promoter to recruit the MeCP1 complex and repress transcription (TF – transcription factor; CH3 – methyl group; HDAC – histone deacetylase; Mbd – methyl binding domain protein; NuRD – Nucleosome remodelling & histone deacetylation complex)

Human genetic studies suggest that 20% of base substitutions causing disease result from the conversion of methylcytosine to thymine (Krawczak *et al.*, 1998). These point mutations are also implicated in the development of some cancers, while *Mbd4*$^{-/-}$ mice have increased rates of tumorigenesis (Millar *et al.*, 2002). In addition to the Mbd family, there is a further binding protein termed Kaiso which is capable of methylation dependent repression of gene transcription. Although it is not an Mbd containing protein, it is capable of binding to methylated DNA via its zinc finger (Prokhortchouk *et al.* 2001). Kaiso has been shown to be a vital component of amphibian development, blocking translation of this protein is lethal (Ruzov *et al.* 2004) but the extent of its role in mammalian systems has yet to be established. Methylation-dependent transcriptional repression is covered by many good reviews such as Wade (2001) and Li (2002).

Transgenic studies show that mice lacking *Mbd1* (*Mbd1*$^{-/-}$) have no observable phenotype, although problems within the nervous system are evident at the molecular level (Zhao *et al.* 2003). Mice homozygous for a disrupted *Mbd2* gene (*Mbd2*$^{-/-}$) mice are also viable, although they exhibit impaired maternal behaviour (Hendrich *et al.* 2001). *Mbd3* null mutations (*Mbd3*$^{-/-}$) are embryo lethal (Hendrich *et al.* 2001). The abnormal phenotype of *MeCP2*$^{-/-}$ mice develops from several weeks of age and is lethal by 8 weeks of age, with all known abnormalities having their origin in the nervous system (Guy *et al.* 2001). The fact that *Mbd1*$^{-/-}$, *Mbd2*$^{-/-}$ and *MeCP2*$^{-/-}$ mice have no apparent phenotype outside of the nervous system suggests there is a degree of redundancy within the Mbd mediated system of transcription control. Although a mouse containing both disrupted *Mbd2* and *MeCP2* genes has demonstrated that both

these proteins function in separate pathways, this does not rule out co-operation between other members of the Mbd family (Guy *et al.* 2001).

1.13 DNA METHYLATION IN GERM CELLS AND EMBRYOS

1.13.1 Primordial Germ Cells

The epigenetic dynamics of the PGCs and gametes are reviewed by Allegrucci *et al.* (2005). When PGCs are first seen in the mouse embryo at E7 they, and the surrounding somatic cells, carry the maternally and paternally inherited imprinting patterns. This DNA methylation pattern is maintained in PGCs as they migrate to the developing gonad. Co-incident with their arrival in the gonadal ridge, the mouse PGCs begin to undergo global demethylation from around E11.5 to remove their inherited imprinting pattern (Hajkova *et al.*, 2002). During this period, DNA methylation of the somatic cells is maintained (Figure 1.9). Demethylation of imprinted gene loci in germ cells is clearly vital if the correct sex-specific epigenetic information is to be subsequently laid down during oocyte and sperm maturation. Demethylation is complete by E13-14, correlating to the period when the male and female mouse PGCs begin to enter mitotic and meiotic arrest respectively. However, there are some repetitive sequences present in the PGCs which do not become entirely demethylated (Hajkova *et al.*, 2002). It has been suggested that mitotic/meiotic arrest might necessarily follow demethylation because replication of unmethylated DNA has an increased risk of unrepressed retro-transposons moving and causing mutations (Walsh *et al.* 1998). The time at which this demethylation occurs, and also the amount of methylation lost, appears to be identical regardless of

the gender of the embryo (Hajkova *et al.* 2002). Whether the loss of methylation occurs by a passive or active mechanism or a combination of both is not yet known, although the speed with which this occurs would suggest involvement of an active mechanism.

As previously described the PGCs of mammals undergo demethylation during E12.5-13.5 as they migrate to the primitive gonad. Although the imprinted genes lose their methylation patterns to allow for the subsequent gender specific patterns of DNA methylation to be laid down in the germ cells the repetitive sequences retain a significant amount of their DNA methylation in PGCs (Marchal *et al.*, 2004). A detailed study of the methylation levels of several classes of repeat sequences have allowed a more thorough understanding of the methylation dynamics of these regions in the PGCs. At E12.5 the IAPs of the female PGCs do not show any substantial demethylation however by E15.5 lower methylation levels were detected at these regions (Lees-Murdock *et al.*, 2003). When L1 elements were assessed they had lower methylation levels at E12.5 than the IAP and the same pattern of methylation loss by E15.5 was observed. The minor satellite sequences had the lowest methylation levels of any of the repeat sequences that were analysed at E12.5 (Lees-Murdock *et al.*, 2003). Again the same dynamic of DNA methylation loss by E15.5 was observed and in all the repeat sequences examined the lowered levels of DNA methylation were maintained at E17.5.

1.13.2 Oocyte Development

In female embryos the gonad forms as an ovary with germ cells forming primordial follicles. As long as the primordial follicle and the oocyte contained within it are not activated to enter the growing population, the methylation level of the oocyte genome remains low and unchanged. It is during the growth phase of the oocyte that the maternal imprints are laid down on the genome (Figure 1.9). The imprints are not all established at the same time: instead, each imprinted gene has a specific time at which it will become methylated (Obata & Kono, 2002; Lucifero *et al.*, 2004). Obata & Kono (2002) analysed parthenogenetic embryos created by nuclear transfer of oocyte nuclei from different stages of follicle development, with the aim of establishing the timing of the maternal imprinting within the oocytes, and showed that *Snrpn*, *Znf127* and *Ndn* genes are imprinted early in follicle development during the primordial to primary follicle stages whilst imprinting of *Peg3*, *Igf2r* and $p57^{KIP2}$ happens at the secondary follicle stage. There are also genes which become imprinted at even later stages of follicle development, including *Peg1/Mest* during tertiary to early antral stages and *Impact* which only becomes imprinted in the oocyte within an antral follicle (Obata & Kono 2002). A further study by Lucifero *et al.* (2004) investigated the methylation of imprinted genes by dissecting follicles from ovaries of different postnatal ages and examining the oocytes. This confirmed the earlier Obata & Kono study in that *Peg3*, *Igf2r* and *Snrpn* began to gain methylation earlier in development that *Peg1*. By the early antral stages some differentially methylated regions (DMRs) were fully methylated in all genes other than *Peg1*, while it was not until oocytes were fully mature that *Peg1* appeared to undergo rapid de novo methylation. Thus, the imprinting pattern of the oocyte is not fully laid

down until it is within a mature follicle ready to ovulate. This has clear implications for ARTs, where follicle and oocyte maturation is usually artificially stimulated; any such process must support the correct completion of oocyte imprinting.

Over the period of oocyte growth, the general level of DNA methylation increases as both the appropriate maternal pattern of imprinting is laid down and non-imprinted sequences also become methylated. The Dnmt involved has yet to be identified, although it has been suggested that one or more members of the Dnmt3 family could be responsible. *Dnmt3a*, *3b* and *3l* are all expressed during postnatal oocyte growth. *Dnmt3l* is expressed at a higher level than either *Dnmt3a* or *Dnmt3b* although all three genes have maximal expression levels occurring at approximately the same stage of oocyte development (Lucifero *et al.* 2004). Dnmt1s protein is not found in either growing oocytes or in pre-implantation embryos. Instead, an alternatively spliced more stable transcript, Dnmt1o, is expressed at these stages. In the growing oocyte, Dnmt1o is found in both the cytoplasm and the germinal vesicle, but once the oocyte is fully matured, it is localised to the cytoplasm where it is stored until it is required during later embryo development (Carlson *et al.* 1992; Mertineit *et al.* 1998). Since Dnmt1o translation only occurs early in oocyte development, the stability of this form of Dnmt1 is clearly important.

The methylation dynamics of the imprinted gene loci during oocyte development are gradually being elucidated. However, the same is not true of the non-imprinted sequences over the same time period. However work has shown that IAPs and L1s are heavily methylated in the mature oocyte (Lees-Murdock *et al.*, 2003). The IAPs

examined had methylation at 66% of the cytosine residues, the transposable element, Etn had 78% of the investigated cytosines that were methylated (Kim *et al.*, 2004). This was contrasted by the centromeric satellite sequences of mature oocytes which had a relatively low level of DNA methylation (21%) compared to the somatic cells (Kim *et al.*, 2004). Dnmt3l does not have the active transmethylase activity which is a characteristic of the other Dnmt3 family proteins, Dnmt3a and 3b. However, Hata *et al.* (2002) found that *Dnmt3l* expression was vital if normal maternal imprints were to be laid down in the oocyte and that this function may be mediated through its ability to bind and co-localise with both Dnmt3a and 3b. Mice with a disrupted *Dnmt3l* gene are sterile. Males produce no mature sperm (see below); females undergo apparently normal oocyte growth and the resulting oocytes can be fertilised, but the absence of maternal *Dnmt3l* is embryo lethal to heterozygote offspring by E9.5 (Bourc'his *et al.* 2001). Interestingly, a conditional knockout with disrupted *Dnmt3a* in the germ cells has an almost identical phenotype to the *Dnmt3l*$^{-/-}$ mouse (Kaneda *et al.* 2004): when females whose oocytes lacked *Dnmt3a* were crossed with wildtype males all offspring died by E11.5, with embryos lacking methylation on the normally methylated maternally imprinted genes (resulting in inappropriate gene expression). This study demonstrates the essential role of Dnmt3a in the establishment of maternal imprints. Kaneda *et al.* (2004) also investigated the role of Dnmt3b using a conditional knockout: these animals were found to be phenotypically normal and were able to produce viable offspring.

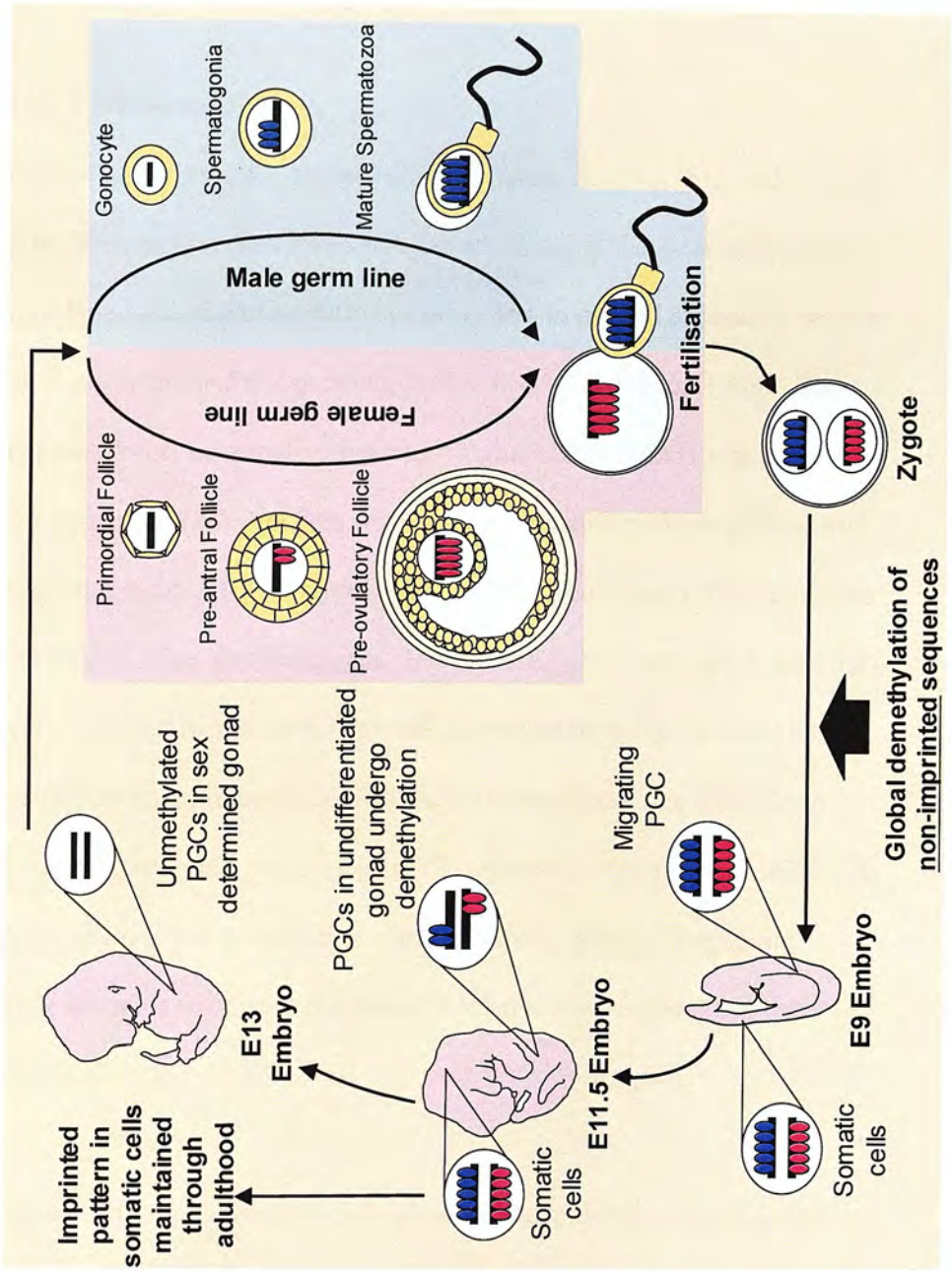


Figure 1.9:- Epigenetic reprogramming of the germ cells. The maternal (pink shaded region) and paternal (blue shaded region) imprints are laid down during germ cell development, such that by the time the oocyte and sperm are fully mature the correct pattern of DNA methylation is present on the genome (female imprints – pink ovals; male imprints – blue ovals). After fertilisation, (yellow shaded area) both parental genomes undergo global demethylation of non-imprinted sequences: imprinted genes are protected from this process. During early embryo development the imprinted genes of both the somatic and primordial germ cells (PGC) retain the parental imprints. From Embryonic Day (E) 11.5 the PGCs begin to undergo demethylation to erase the inherited parental imprints, but the somatic cells of the embryo maintain the parental imprints through embryo development and into adulthood. The process of PGC demethylation is complete by E13. Subsequent reprogramming of the germ cells occurs when the gender-specific imprinting patterns are once more laid down. Hajkova et al., 2002; Kerjean et al., 2000; Oswald et al., 2000; Ueda et al., 2000; Santos et al., 2002; Lucifero et al., 2004; reviewed by Allegrucci et al., 2005.

1.13.3 Sperm Development

As with the oocyte, new imprints are laid down as sperm develop (Figure 1.9), with the increase in DNA methylation levels not just attributed to the establishment of paternal imprints but also the methylation of other non-imprinted sequences, such as intracisternal A particles (IAPs), becoming methylated (Walsh *et al.* 1998). The paternally expressed (i.e. maternally imprinted) human *MEST/PEG1* gene is demethylated during fetal life and then remains unmethylated through all stages of sperm development in adult life (Kerjean *et al.*, 2000). Ueda *et al.* (2000) analysed the methylation level of an imprinted gene, *H19*, in male germ cells and found that the *H19* imprint is laid down early in germ cell development before meiosis occurs. The same result was found in humans, with the *H19* gene becoming methylated before meiosis at the spermatogonial stage of development (Kerjean *et al.* 2000). In general, though, there is less information about the laying down of imprinting patterns during sperm development compared to what is known about imprinting in oocytes.

The resumption of mitotic division of male germ cells at puberty coincides with an increase in the level of *Dnmt1* within the spermatocytes. During the early stages of meiosis the level of Dnmt1s in spermatocytes is high but a reduction in the level of the Dnmt1 enzyme has been observed in pachytene stage spermatocytes (Jue *et al.* 1995). This is due to the expression of an alternatively spliced version, *Dnmt1p*, which does not appear to be translated. Although Dnmt1s within the sperm is normally found in both the nucleus and the cytoplasm, it is concentrated at nuclear foci during some stages of meiosis and it may be that this correlates with the laying

down of paternal imprints (Jue *et al.* 1995). *Dnmt3l* is expressed in the murine testes from E12.5 in non-dividing prospermatogonia with peak expression seen at the time of birth, after which there is a dramatic post-natal reduction in expression level (La Salle *et al.* 2004; Bourc'his & Bestor 2004). *Dnmt3a* expression in the testis is raised before birth and during early postnatal life, in contrast to the level of *Dnmt3b* expression which is lower during embryonic life and rises postnatally (La Salle *et al.* 2004). Mice lacking *Dnmt3l* have smaller testes, and by adulthood there are virtually no spermatozoa present resulting in sterile animals (Hata *et al.* 2002). Dnmt3l is required if normal meiosis and silencing of retrotransposons is to occur (Bourc'his & Bestor 2004). The loss of *Dnmt3a* results in a similar although less extreme phenotype than that seen in the *Dnmt3l*$^{-/-}$ mouse (Hata *et al.* 2002). More recently the male *Dnmt3a* conditional knockout was created, with no germ cell *Dnmt3a* expression but with somatic cell levels maintained (Kaneda *et al.* 2004). Spermatogenesis is severely impaired in these mice so that by 11 weeks of age there are no spermatozoa in the testis, demonstrating a vital role for Dnmt3a in this process. Offspring from these conditional knockout males have errors in the methylation of some paternally imprinted genes.

1.13.4 Fertilisation and Early Embryo Development

As shown in Figure 1.9, the fertilised embryo contains methylated DNA some of which will be located in imprinted genes (both maternal and paternal) while the majority of the DNA methylation will be positioned on non-imprinted sequences (again of both maternal and paternal origin). Early on in embryo development, the embryo loses its methylation at the non-imprinted DNA sequences. It now appears

that this DNA methylation is lost in a parent-of-origin specific order, at least in some species. Imprinted genes are resistant to these early demethylation processes. The embryo's germ cells will lose methylation of the imprinted genes during gonadal development, while somatic cells maintain these methylation patterns throughout embryonic development and, in the main, throughout the life of the newly formed organism, (although imprinted patterns are lost or altered in some tissues, such as the liver; McLaren & Montgomery 1999).

In some species (such as the mouse), the paternal genome is actively demethylated immediately after fertilisation (Oswald *et al.* 2000). The occurrence and degree of this demethylation appears to be species-specific, and its regulation is currently unknown. Cross-fertilisation using gametes from several species of animal has found that although there are sperm characteristics which affect the degree of demethylation, the main factor determining whether the paternal genome becomes demethylated is oocyte-specific (Beaujean *et al.* 2004c). In the search for the factor responsible for this post-fertilisation active demethylation, Mbd2 was proposed as a candidate after an *in vitro* study by Bhattacharya *et al.* (1999). However, a subsequent study utilising *Mbd2*$^{-/-}$ oocytes has found that the rapid demethylation of the paternal genome still occurs in its absence (although this result doesn't eliminate the possibility of redundancy) (Santos *et al.*, 2002). The maternal genome undergoes passive demethylation which is slower to occur and is linked to the replication of DNA in the absence of any maintenance methylase activity. Although the general trend after fertilisation is for non-imprinted sequences to undergo demethylation,

there does appear to be some specific incidences of de novo methylation such as the DMRs of the *Dnmt1o* gene in the 1-cell to blastocyst stage embryo (Ko *et al.* 2005).

Dnmt1o remains localised to the cytoplasm of the embryonic cells at all pre-implantation stages with the exception of the 8-cell embryo. During this stage, the protein has been shown to translocate to the nuclei, where it is thought to play a role in maintaining the methylation level of imprinted genes (Carlson *et al.* 1992; Mertineit *et al.* 1998; Ratnam *et al.*, 2002). *Dnmt3a* is expressed by pre-implantation embryos and there is no evidence of the protein being excluded from the nucleus at any developmental stage (Ko *et al.* 2005). Although *Dnmt3b* is not transcribed in the pre-implantation embryo the protein is present at all stages from the 1-cell to blastocyst: while mainly localised to the cytoplasm, it is not fully excluded from the nucleus (Ko *et al.* 2005).

Until recently embryos which consisted of two maternal or two paternal genomes were unable to develop to term. Work examining the competence of parthenogenetic embryos found they were able to develop at best until E9.5. By creating an embryo with one set of chromosomes from a fully grown and the other from a non-growing oocyte, Kono *et al.* (1996) showed that embryo development could be extended to E13.5. It was thought that this increase in the length of time the embryo survived was due to the ability of the non-growing oocyte chromosomes (with, therefore, no female imprints yet laid down) to partially compensate for the lack of a paternally imprinted set of chromosomes. Parthenotes which died at E9.5 and E13.5 had incorrect biallelic expression of the normally monoallelic *H19* gene. The most recent

work by Kono *et al.* (2004) has resulted in the birth and survival of a viable parthenote. This was achieved using non-growing oocytes from a transgenic mouse with a 13-kilobase deletion of *H19*. The mouse had *H19* expression consistent with that normally seen from the paternal genome. As *H19* in turn influences the expression of the imprinted *Igf2* gene, the embryo had monoallelic expression of both *H19* and *Igf2* (from the wild type fully grown oocytes only), as would be the case in normal embryos. The fact that normal embryo development is possible after the female genome is altered to more closely resemble the genomic imprinting pattern of the male genome demonstrates the importance of this mechanism in controlling development, reinforcing the idea that the presence of mammalian genomic imprinting might act as a barrier to parthenogenesis (see above).

1.13.4.1 X-Inactivation

The process of random inactivation of one of the two X-chromosomes in the somatic cells of the early female embryos is a vital developmental process. DNA methylation has an established role in ensuring that X-inactivation occurs correctly and that long-term silencing of gene transcription is maintained (Reviewed by Ehrlich, 2003a). However, DNA methylation is not required to initiate the silencing of the inactive X-chromosome (Xi) and is in fact necessary for protecting the X-chromosome that is to remain active. The mechanism by which Xi is silenced involves the transcription of *Xist* which is an untranslated but functional RNA which coats the *Xist* expressing chromosome causing gene inactivation. The key role of DNA methylation in this process is on the active X-chromosome as on this chromosome the *Xist* 5' region is methylated to prevent transcription of the *Xist*

RNA. Once inactivation of the X-chromosome has been initiated DNA methylation plays a role in ensuring these silenced genes are not reactivated but methylation. Thus, methylation functions to maintain rather than initiate the gene silencing on Xi (Reviewed by Bird, 2002).

1.14 ERRORS IN GENOMIC IMPRINTING

With genomic imprinting being a basic mechanism clearly vital for many aspects of development, there are, not surprisingly, many instances of developmental defects due to imprinting errors whether occurring naturally or during human intervention (Table 1.4).

1.14.1 Assisted Reproductive Techniques

In recent years, there has been increasing concern that children conceived with the aid of ARTs could have increased occurrence of disorders linked to imprinting problems. At the turn of the century, two studies (Cox *et al.* 2002; Orstavik *et al.* 2003) reported the occurrence of three children conceived using intra cytoplasmic sperm injection (ICSI) with Angelman Syndrome (AS), a neurological disorder characterised by developmental delay and seizures, suggesting that the risk of AS may be increased by the use of ICSI. The fear of such a link was then increased with three studies that examined patients with Beckwith-Wiedemann Syndrome (BWS) to see if a higher than expected proportion of these cases came from ART babies; all studies found a disproportionate number of such cases (DeBaun *et al.* 2003; Maher *et al.* 2003; Gicquel *et al.* 2003). BWS is characterised by both pre- and post-natal

overgrowth and defects of the abdominal wall. Children who had been conceived using ART and suffered from BWS had the methylation status of their *H19* and *LIT1* genes established, with only one of the identified children demonstrating normal methylation patterns on both these genes. Data suggest that ART results in a three- to six-fold increase in the incidence of the normally rare BWS, although some of the studies may in fact be underestimating the true risk (DeBaun *et al.* 2003). However, a link between ART and imprinting disorders has not been definitively confirmed, one large scale Danish study (involving 442 349 non IVF and 6053 IVF children) found no incidences of imprinting disorders amongst the children conceived by ART (Lidegaard *et al.*, 2005). Thus, the apparent link identified by earlier studies (Cox *et al.* 2002; Orstavik *et al.* 2003 DeBaun *et al.* 2003; Maher *et al.* 2003; Gicquel *et al.* 2003) could be due to the statistical approach required for such small studies (Gosden *et al.*, 2003). The cause of the link between ART and imprinting disorders is currently unknown. It could be due to some aspect of the ARTs involved. There is a wide range of different ARTs which are now routinely used within clinics. Techniques might expose one or both of the germ cells to an altered hormonal regime *in vivo*, a period of time in culture or mechanical manipulation. Any such alterations to the normal environment of the oocyte or sperm could result in changes to some aspect of their imprinting mechanisms. Alternatively, it could be due to some error within the germ cells used, bearing in mind that couples seeking to use ARTs have reduced natural fertility.

The potential problems do not end with the germ cells; the pre-implantation embryo is also often exposed to a period of culture which could again alter the epigenetic

reprogramming known to occur at these early stages. One such example is abnormal biallelic *H19* expression of mouse embryos cultured in Whitten's medium (Doherty *et al.* 2000). It is not just in humans and mice that potential problems with imprinting have been seen. In large domestic mammals such as sheep and cattle, Large Offspring Syndrome (LOS) was identified when embryos had been exposed to some time in culture (Young *et al.* 1998). Further investigations into LOS in sheep have identified changes in the expression level of the imprinted gene, *IGF2R* due to epigenetic changes (Young *et al.* 2001). Similar overgrowth problems seen in mice and humans are often caused by errors in several different imprinted genes including *Igf2* and *H19* (Eggenschwiler *et al.* 1997).

If sperm used for *in vitro* fertilisation (IVF) have lowered global methylation levels there is no alteration in either fertilisation rate or in early embryo quality, however there is a reduction in pregnancy rate demonstrating the importance of normal gamete DNA methylation on embryo development and ultimately ART outcome (Benchaib *et al.* 2005). The analysis of sperm DNA methylation levels was carried out using flow cytometry of sperm suspension which had been stained with 5-methylcytosine antibody (Benchaib *et al.*, 2005). The results obtained represent the mean methylation levels of the sperm population present in each semen sample (Benchaib *et al.*, 2005).

Table 1.4:- Diseases and syndromes resulting from failures in genomic imprinting, DNA methylation or errors in DNA methylation mechanism.

DISORDER	AFFECTED GENES	PHENOTYPE	ART LINK	REFERENCE
IMPRINTING DISORDERS				
Angelman Syndrome	Chromosome 15 – maternal copy, loss of *SNRPN* imprinting	Mental retardation, ataxic gait, seizures, sociable disposition	✓	Buiting et al., 1998 Polychronakos & Kukuvitis, 2002
Beckwith-Wiedemann Syndrome	11p15 region – altered expression of *IGF2*, *H19* & *LIT1*	Undescended testes, large newborn, seizures, abdominal wall defects	✓	Maher & Reik, 2000 Weksberg et al., 2003
Prader-Willi Syndrome	Chromosome 15 – paternal copy	Undescended testes, Mental retardation, short stature, obesity, small hands & feet		Buiting et al., 1998 Polychronakos & Kukuvitis, 2002
Pseudohypoparathyroidism type IA (Albright hereditary osteodystrophy)	Imprinted *GNAS* cluster	Parathyroid hormone resistance, short stature, round face & short hand bones		Davies & Hughes, 1993 Polychronakos & Kukuvitis, 2002
Pseudohypoparathyroidism type IB	Imprinted *GNAS* cluster	Parathyroid hormone resistance localised to renal system causing hypocalcemia & hyperphosphatemia		Liu et al., 2000 Polychronakos & Kukuvitis, 2002
Silver-Russell Syndrome	Cases which are imprinting related - Chromosome 7	Short stature, excessive sweating, triangular face, inward curving 5th fingers & coloured spots on the skin.		Hitchins et al., 2001 Polychronakos & Kukuvitis, 2002
Transient neonatal diabetes	An imprinted gene at 6q24. Candidates are *ZAC* & *HYMA1*	Growth retardation & diabetes which develops during the first 6 months of life but corrected by 18 months old		Gardner et al., 2000 Varrault et al., 2001
Wilms' tumor	*IGF2* loses imprinting	Childhood kidney tumour		Frevel et al., 1999
METHYLATION (SOME CASES MAY BE LINKED TO IMPRINTING FAULTS)				
Autism	Unknown X-linked gene (not always connected to imprinting)	Impaired language development, problems with social & motor skills		Carney et al., 2003
Cancer	Variable e.g. *IGF2* in lung cancer (not always connected to imprinting)	Tumours		Szyf, 2003 Mizuno et al., 2001
Paraganglioma	Paternal mutations *SDHD* (*PGL1*) & *PGL2*	Glomus tumours of parasympathetic ganglia mainly in head & neck region, tend to be slow growing & benign		Baysal, 2004
Pre-eclampsia	Not yet defined but suggested to involved imprinted genes in some families	Serious complication of pregnancy		Constância et al., 2004
Turner Syndrome	Complete or partial loss of second X chromosome	Affects females - short stature, social problems & ovarian failure		Gravholt, 2004
FAILURES IN THE METHYLATION/IMPRINTING MECHANISM				
ICF	*DNMT3b*	Immune problems, facial anomalies, growth retardation		Ehrlich, 2003
Rett Syndrome	*MECP2*	Childhood neurodevelopmental disorder mainly affecting females. Loss of motor functions & mental retardation		Amir et al., 1999 Kriaucionis & Bird, 2003

There is recent evidence that sperm obtained from males with low sperm counts due to abnormal spermatogenesis have incorrect genomic imprinting (Marques *et al.* 2004), although such sperm can then be used e.g. in ICSI. Marques *et al.* (2004) found that although the maternal imprints had been erased from all sperm, the paternally methylated *H19* gene was under-methylated in some sperm from the oligozoospermia donors. Any embryo derived from one of these hypomethylated sperm could have inappropriate expression of the imprinted *H19* and *IGF2* genes, the effect of which is not known.

1.14.2 Somatic Cell Nuclear Transfer

Studies investigating the failure of animals created by somatic cell nuclear transfer have also turned their attention to the role of genomic imprinting. The fact that many of the errors seen in these animals have epigenetic causes has been demonstrated by examining the offspring of mice born as a result of somatic cell nuclear transfer. The mice obtained using somatic cell nuclear transfer were obese but this trait was not passed onto the offspring demonstrating that this was not a genetic error but due to epigenetics. This finding is important as it suggests that, despite any problem in animals derived using somatic cell nuclear transfer, it is possible that their germ cells are able to correctly undergo genomic imprint reprogramming (Tamashiro *et al.* 2002). In bovine embryos produced using somatic cell nuclear transfer it has been found that the levels of methylation in the cells of the embryo are higher than normal at the 4-cell and 8-cell stages. Although there is initial demethylation of the donor genome, passive demethylation does not occur to the level seen in normal embryos. In addition to a reduction in the amount of demethylation there also appears to be

inappropriate de novo methylation occurring at early stages of embryo development (Dean *et al.* 2001). It is also possible that errors in the Dnmt enzymes normally present in the early embryo could account for alterations in methylation seen in these embryos. Analysis of mouse embryos resulting from somatic cell nuclear transfer shows inappropriate presence of Dnmt1s within the pre-implantation embryo; this isoform of Dnmt1 is never present in normal embryos. It was also observed that at the 8-cell stage, when Dnmt1o would normally translocate into the nuclei of embryonic cells, some nuclei within each embryo were devoid of any Dnmt1 isoform suggesting that these cells are unable to maintain normal methylation patterns (Chung *et al.* 2003).

1.14.3 Disease

In some cases imprinting errors can occur which, although not embryo lethal cause abnormal physiological processes and lead to disease. Such diseases can arise when any imprinted gene becomes hypermethylated or hypomethylated. Effects are not always limited to the loss of function of a single gene, as some imprinted genes affect the expression of other genes, such as *H19* and *IGF2*. The linked Prader-Willi Syndrome (PWS) and AS are examples of disorders that can occur when correct imprinting is lost. A loss of a currently unidentified imprinted gene results in PWS when the deletion is paternally inherited, whereas the same errors cause AS to develop when maternally transmitted (Moncla *et al.* 1999). Other examples of diseases which result after incorrect imprinting include BWS, Silver-Russell Syndrome and transient neonatal diabetes.

Disease can also result from defects in mechanisms regulating imprints. One of the key groups of enzymes with a role in genomic imprinting are the Dnmts which are responsible for the addition of methyl groups to the DNA. When problems arise within this aspect of the imprinting mechanism it can lead to disease in the individual. One such example is immunodeficiency, centromeric region instability, and facial anomalies syndrome (ICF) which is a result of a mutation in *DNMT3b* (Reviewed by Ehrlich 2003b). Another major component of the imprinting mechanism is the family of methyl binding domain proteins. MeCP2 is a protein which contains a methyl binding domain. It has a role is controlling the transcription of imprinted genes through its ability to bind to methylated DNA. The importance of this protein for normal development and physiological function is demonstrated by Rett syndrome which occurs when *MECP2* is mutated (Amir *et al.* 1999).

There are some diseases with multiple causes which only in some cases involve errors to the imprinting mechanism or alterations to imprinted genes. Cancer is one such disease, with some cases of cancer being identified as having a cause linked to genomic imprinting while many other incidences of the disease occurring due to unrelated problems. In some instances, human tumour cells have been found to overexpress one or more of *DNMT1s*, *3a* and *3b*, with the largest upregulation occurring to *DNMT3b* (Robertson *et al.* 1999). These results support the previous observations of abnormal methylation levels seen in tumour cells. One cancer which demonstrates such raised DNMT levels is acute myelogenous leukaemia; it may be that this over-expression of the DNMT enzymes accounts for the hypermethylation and silencing of an important tumour suppressor gene (Mizuno *et al.* 2001).

1.15 PROJECT BACKGROUND

The study described in this section and some of the data presented within this thesis form the basis of a joint first author paper. Throughout this thesis the reference is cited as Murray *et al.* (2005) (Appendix G).

In previous work by the laboratory, oocytes were exposed to altered levels of androgens and oestrogens in culture to investigate their effect on follicle development and oocyte maturation. The subsequent developmental competence of these oocytes was then assessed using IVF. The *in vitro* method used was a whole follicle culture system capable of supporting follicular development of isolated pre-antral follicles until the Graafian stage (Spears *et al.*, 1994). Follicles were exposed to altered steroidal regimes *in vitro* for the duration of the culture period before oocytes were recovered and used in IVF. Exposure to raised androgen levels (Figure 1.10A) were achieved through the addition of Arimidex, a potent inhibitor of aromatase activity, to the medium; this also resulted in a significant reduction of oestradiol production by the follicles due to the loss of aromatisation of androgens to oestrogens (Figure 1.10B). At present, only the concentration of androstenedione has been assayed; it may also be interesting to determine the levels of testosterone or DHT produced by the follicles cultured in the presence of Arimidex. Oestrogen levels were increased through the medium being supplemented with the synthetic oestrogen, diethylstilboestrol (DES). However, DES is not able to cross-react with the highly specific oestradiol assay thus the additional oestrogenic activity of this synthetic oestrogen cannot be measured using the assay (Figure 1.10B). The final treatment group consisted of high levels of androgen and oestrogen in combination.

The above alterations to the steroidal environment had no effect on either the growth rate of the follicles, levels of apoptosis or antral formation. In order to build on this previous study the high steroid treatment culture groups described within this thesis utilise the same Arimidex and DES doses as the original Murray *et al.* study.

Although alteration of the steroidal environment had no apparent effect on follicular morphology there was a significant effect of high steroids on the developmental competence of the oocyte. In IVF experiments, oocytes which had been exposed to raised levels of oestrogen either alone or in combination with raised androgens had significantly reduced fertilisation rates ($P<0.01$) (Figure 1.11). In contrast the oocytes exposed to medium containing only raised androgen levels had an improved rate of fertilisation (Figure 1.11A). However, these effects of steroids did not persist during embryo development with no effect seen in the ability of 2-cell embryos to develop to the blastocyst stage regardless of the steroidal milieu they were exposed to during follicular development (Figure 1.11B). Although the results from this work suggest that raised steroid levels have an effect on oocyte maturation, the mechanism through which they exert this is unknown. As previously described in Section 1.14, DNA methylation of the oocyte genome is a vital component of the maturation process which can be influenced by the environmental conditions. Taking this into consideration it is possible that the observed effects of steroids on fertilisation rate could be mediated through alterations to DNA methylation during oocyte maturation (Figure 1.12).

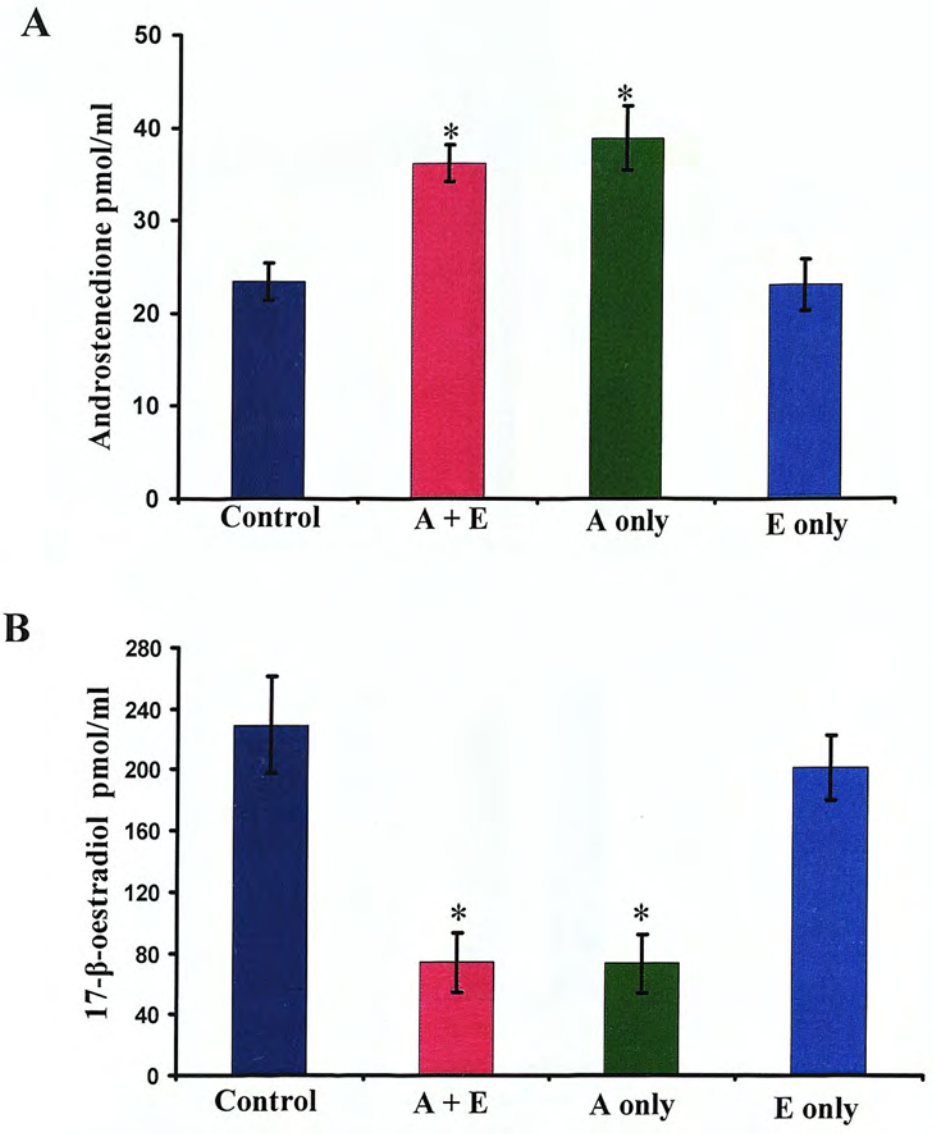


Figure 1.10:- A) Concentration of androstenedione produced by follicles grown in different steroid environments. **B)** Concentration of 17-β-oestradiol produced by follicles grown in different steroid environments. Media was sampled on day 6 of culture. Values are mean ± SEM ($n \geq 9$ for each group). * $p<0.001$.

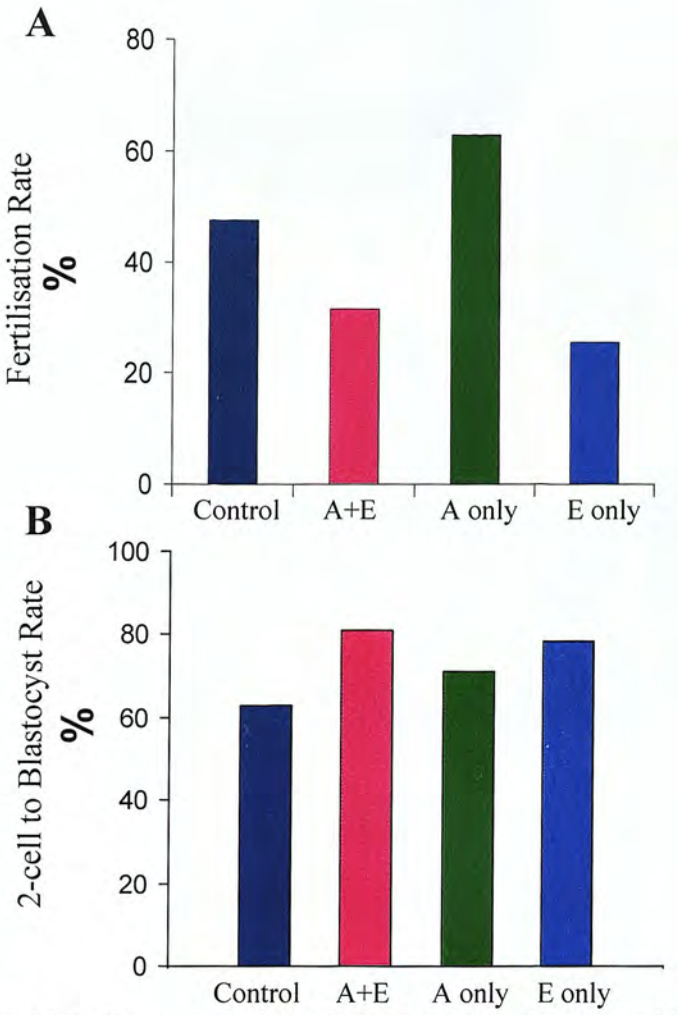


Figure 1.11:- Oocytes which had been exposed to altered steroidal regime during pre-antral and antral development were used for *in vitro* fertilisation (IVF). Experimental treatment groups were: A only – high androgen; E only – high oestrogen; A+E – high androgen and oestrogen in combination. **A)** A significant alteration in fertilisation rate was observed after exposure to altered steroid levels ($P<0.01$). Fertilisation rates were reduced after culture with oestrogen (whether alone or in combination with high androgen). When only androgen levels were raised during culture the oocytes had improved developmental competence, with a higher percentage being fertilised. **B)** There was no effect of steroid environment on the percentage of 2-cell embryos capable of developing to the blastocyst stage. Three repeats of this experimental protocol were carried out. The number of oocytes in each treatment group was as follows – Control, n=57; A+E, n=51; A only, n=49; E only, n=51.

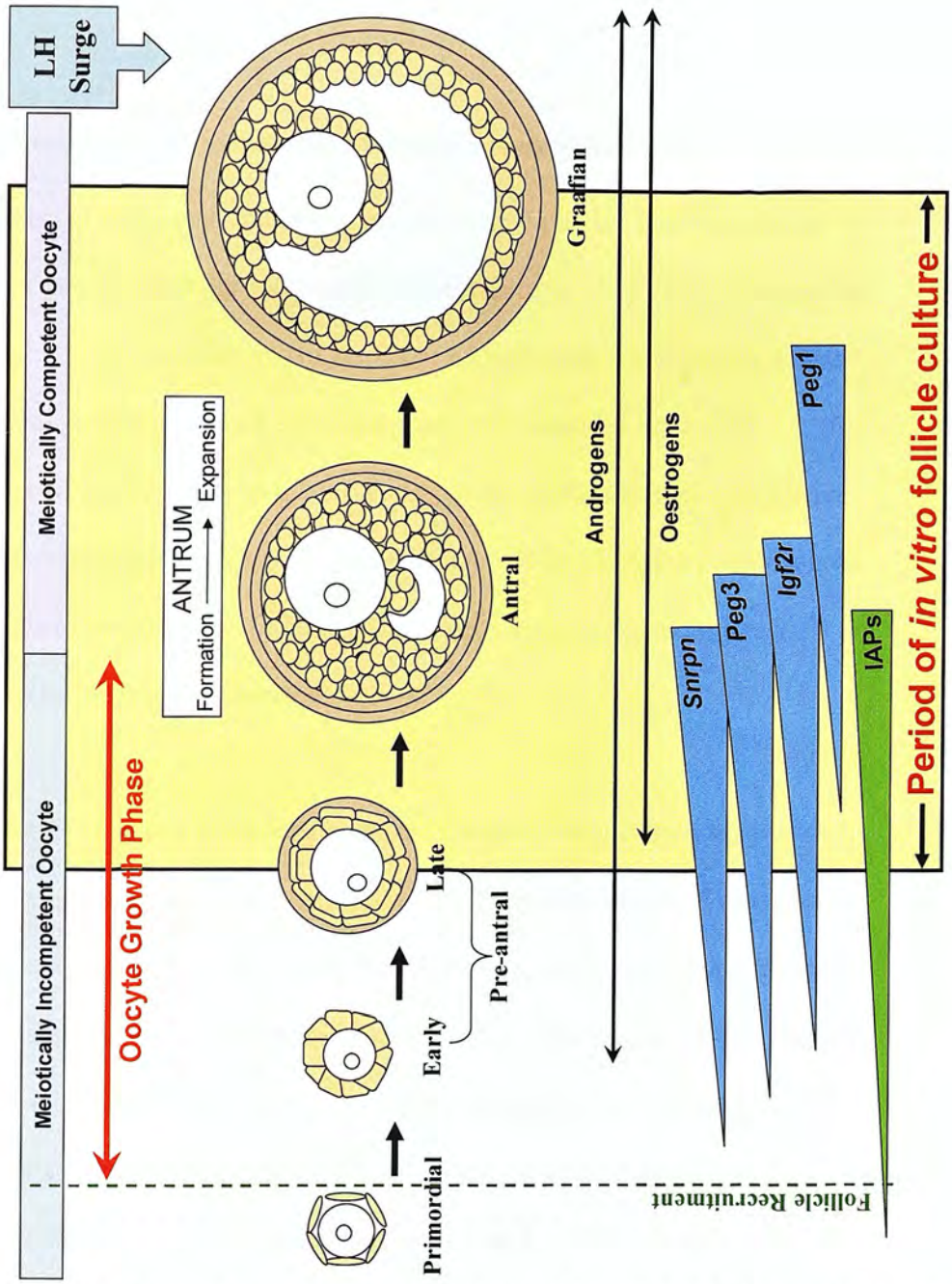


Figure 1.12:- Diagram depicting some of the key processes occurring over the period of oocyte and follicle development. After recruitment (green dashed line) into the growing population of follicles the oocyte of the early pre-antral follicle enters a period of rapid growth, as demonstrated by the red arrow. The oocyte is meiotically incompetent (represented by blue bar) and gains meiotic competence (represented by purple bar) around the time of antrum formation (represented by white bar) as the oocyte achieves its maximum size. Follicular synthesis of oestrogens and androgens are represented by the black arrows extending across the majority of follicle development. Each imprinted gene becomes methylated at a gene-specific time point over oocyte development, ranging from early pre-antral to late antral stages. The yellow shaded region represents the period of follicle development that correlates to that seen in the follicle culture system used in the experimental chapters of this thesis. The oocytes within pre-antral follicles at the start of culture have not yet completed their growth, are meiotically incompetent and will have incomplete maternal DNA methylation patterns. (Spears et al., 2004; Murray & Spears, 2001; Obata & Kono 2002; Lucifero et al., 2004; Mehlmann, 2005)

Yellow cell - granulosa cells. Brown cell layers - theca cell layers. Triangles represent increasing DNA methylation of imprinted genes (blue) and Intracisternal A particles (IAPs) (green).

1.16 RESEARCH AIMS

The importance of androgens and oestrogens for normal ovarian function and the production of developmentally competent oocytes has been a key area of research for several decades. However, the role of these steroid hormones is still widely debated, with conflicting results as to their influence on the mammalian oocyte. The increasing use of ARTs means an understanding of the mechanisms by which these hormones exert their influence has become ever more vital. Thus, the principal goal of this thesis is to investigate the effects of androgens and oestrogens on oocyte maturation using the mouse as a model.

It has been established that alterations in genomic imprinting and DNA methylation, can negatively influence the developmental competence of the oocyte. There is an accumulation of evidence, which suggests that altered genomic imprinting can result from exposure of the oocyte or embryo to altered environmental conditions. There is conflicting evidence as to the role of the steroid hormones on the maturation and developmental competence of the mammalian oocyte. There is a correlation between the timing of oocyte exposure to altered levels of steroids, such as after the administration of superovulatory exogenous gonadotrophins, and the vital process of DNA methylation of the oocyte genome (Figure 1.12). Therefore, it is possible that changes to the level or pattern of androgen and oestrogen exposure could result in aberrant DNA methylation and have an impact on subsequent gene expression. Thus, this thesis will investigate the effects of androgens and oestrogens on mammalian oocyte maturation.

More specifically this thesis will aim to further the understanding of the methylation dynamics of the oocyte by:-

- Analysing the expression of key enzymes involved in control of imprinted gene expression (Chapter 3)
- Investigating changes to the methylation dynamics of the oocyte in response to steroids (Chapter 4 and 5)
- Investigating the reproduction of the *Mbd2*$^{-/-}$ mouse (Chapter 6)

Chapter 2

Materials & Methods

2.1 Animals

All animals used in Chapters 3, 4 and 5 of this thesis were F1 hybrids from a C57Black/6J x CBA background while Chapter 6 used mice with a C57Black/6J background in addition to the F1 animals. All mice were housed in environmentally controlled rooms with 12 hour light: 12 hour dark photoperiod with both food and water freely accessible. All animals were kept in accordance with UK legal requirements.

2.2 Dissecting Medium

The osmolarity of Leibovitz L-15 medium (Invitrogen, UK) was adjusted to within the range 285-293mOsmKg^{-1} using sterile hospital grade water (Phoenix Pharmaceuticals, USA). 3mgml^{-1} of Bovine Serum Albumin (BSA, Fraction V, tissue culture grade, Sigma-Aldrich, UK) was added to the Liebovitz and allowed to dissolve prior to filter sterilising (syringe – Becton Dickinson Biosciences, UK; filter – 0.22μm cellulose acetate filter, Iwaki). The dissecting medium was warmed in watchglasses placed in a 37°C oven prior to use. Dissecting medium was freshly made for every experiment and was not kept beyond 8 hours at 37°C.

2.3 Superovulation

Mice were injected intra-abdominally with 0.1ml PMSG (5IU/100µl Phosphate Buffered Solution (PBS), Intervet UK Limited, UK) and 48 hours later a second injection of 0.1ml hCG (5IU/100µl PBS, Intervet UK Limited, UK) was administered using insulin syringes (Sherwood, UK). 12 hours after the second injection the mice were killed by cervical dislocation and the ovaries and oviducts were removed into sterile dissecting medium (as described in Sections 2.2 and 2.4).

2.4 Removal of Ovaries and Oviducts

Female mice were killed by Schedule 1 cervical dislocation. When appropriate the ovaries or ovaries and oviduct were removed through an abdominal incision and immediately placed in warmed (37°C) dissecting medium (as described in Section 2.2). The dissection was carried out in a laminar flow hood (Astecair, BioQuell, UK) to maintain a sterile environment.

2.5 Follicle Dissection (Day 0)

Ovaries were halved and placed in fresh dissecting medium and kept at 37°C until use. Pre-antral follicles, 175 ± 2 µm in diameter, were dissected using an insulin syringe (Sherwood, UK) and a mounted acupuncture needle (Acumedic Limited, UK) under a dissecting microscope (Zeiss, Carl Zeiss Ltd, UK) with heated stage

(Linkam Scientific Instruments, UK) at 37°C. Both the horizontal and vertical diameters of each follicle were measured using an ocular micrometer and the mean of these two values used to determine the follicle size. As the processes of follicle and oocyte development are correlated, follicle size can be used as an accurate stage determinant (Gosden *et al.*, 1993). Follicles were dissected from the ovary as rapidly as possible with each half being worked with for only 30 minutes before disposal. Once dissected out from the ovary follicles destined for culture were transferred to the culture wells within 45 minutes to give them the best chance of survival. In cases where a period of time in culture was not part of the experimental protocol oocytes were recovered from the dissected follicles as rapidly as possible to prevent deterioration.

2.6 Oocyte Recovery

2.6.1 Recovery from Follicles and Fixation

Follicles were placed into M2 medium (Sigma-Aldrich, UK) supplemented with 20μlml^{-1} of dbcAMP (Sigma-Aldrich, UK) to prevent the oocytes spontaneously resuming meiosis when released from the follicular environment. M2 medium was stored at 4°C after opening and disposed of after one month. dbcAMP was added immediately prior to use of the M2 medium and was not stored at the working dilution. Follicles were ruptured using insulin needles (Sherwood, UK) and the recovered COCs were then repeatedly passed up and down a fine BSA coated glass pipette to mechanically remove the cumulus cells from the oocyte. Oocytes were fixed in freshly made 4% paraformaldehyde (PFA, Sigma-Aldrich, UK) overnight at

4°C. After fixation oocytes were carefully removed from the PFA and placed in autoclaved PBS (Sigma-Aldrich, UK) where they could be stored at 4°C for up to a week before use.

2.6.2 Recovery from Oviducts

The ovaries and oviducts were removed post-mortem into sterile dissecting medium (as described in Sections 2.2 and 2.2). The oviducts were ruptured using insulin syringes (Sherwood, UK) and the COCs retrieved. If these oocytes were to be fixed for immunohistochemistry they were transferred into fresh dissecting medium supplemented, immediately before use, with 300μgml^{-1} hyaluronidase (Sigma-Aldrich, UK) to remove the cumulus cells. Oocytes were then fixed in freshly made 4% paraformaldehyde (PFA, Sigma-Aldrich, UK) overnight at 4°C. After fixation oocytes were carefully removed from the PFA and placed in autoclaved PBS (Sigma-Aldrich, UK) where they could be stored at 4°C for up to a week before use. Alternatively if the oocytes were required for IVF no removal of cumulus cells was undertaken and COCs were immediately utilised in the IVF protocol.

2.7 Intact Follicle Culture System

2.7.1 Basic Culture Medium

The main constituent of the culture media used in all the experiments was α-Minimum Essential Medium (α-MEM, Invitrogen, UK). Sterile hospital grade water (Phoenix Pharmaceuticals, Germany) was added to give a final working osmolarity of 285mOsmKg^{-1}. α-MEM was stored at 4°C for a maximum of 7 days once

adjusted to the final working osmolarity. The α-MEM was supplemented with charcoal stripped fetal bovine serum (50μlml^{-1}); 1 IU ml^{-1} rhFSH to support follicle growth (Puregon®, Organon Laboratories Ltd, UK); ascorbic acid (140μm ml^{-1}, Sigma-Aldrich, UK). Charcoal stripping of the fetal bovine serum was carried out in the laboratory using the method of Huot & Shain (1988) in order to remove the endogenous steroids. Ascorbic acid was added to the medium as previous work from our laboratory has demonstrated its positive effect on follicle integrity and survival (Murray *et al.*, 2001). Once all of the components had been added the medium was filter sterilised (syringe – Becton Dickinson Biosciences, UK; filter - 0.25μm, Iwaki, UK).

2.7.2 Culture System

The culture system employed was an intact follicle culture protocol with a single follicle per well. 96 well non-tissue culture treated round-bottomed plates (Iwaki, UK) were used for all cultures. 30μl of medium was aliquoted into the first two rows of wells and covered with 75μl of filtered silicon oil (Merck, Germany) (syringe – Becton Dickinson Biosciences, UK; filter - 0.45μm, Iwaki, UK). Trays were incubated overnight (37°C in a humidified 5% CO_2, 95% air atmosphere incubator, Thermo Electron Corporation, UK).

Trays were incubated (37°C in a humidified 5% CO_2, 95% air atmosphere incubator, Thermo Electron Corporation, UK) with the time outside the incubator being kept to a minimum to ensure the optimum temperature was maintained. Dissected pre-antral follicles (as described in Section 2.5) were placed in the first row of wells on the day

of dissection (Day 0). The following day follicles were transferred into the second pre-prepared row of medium (Day 1). Fresh medium was made and filtered on day 2 (as described in Section 2.7.1), 60µl of medium was placed into each culture tray well and covered with 75µl of silicon oil (Merck, Germany). Plates were returned to the incubator for at least one hour, to allow equilibration of the medium, before follicles were transferred into these freshly prepared wells. The follicles were not moved for the remainder of the culture period. Follicles were examined every day to check their health and growth. Measurements of follicle diameter were carried out as described in Section 2.5 and the health of the follicle was visually assessed.

2.8 Polymerase Chain Reaction (PCR)

The master mix for PCR containing 10X buffer (200mM Tris-HCl (pH 8.4), 500mM KCl, Promega, UK), magnesium chloride (Promega, UK), dNTP mixture (Promega, UK), and platinum *Taq* polymerase (supplied at 5 U/µl, Invitrogen, UK) had the appropriate PCR primers (Eurogentec, UK) added as specified in the relevant Sections 3.3.4 and 6.3.2 (Table 6.1). After the addition of the relevant volume of cDNA or DNA the volume was made up to 25µl per tube with autoclaved distilled water. All components of the master mix were stored at -20°C and were thawed and mixed in the appropriate volumes only when required. The master mix was kept on ice at all times prior to the sterile 0.5ml PCR tubes being loaded onto the PCR machine (M J Research, UK). The concentration of all constituents was optimised for each gene of interest (Section 3.3.4 And 6.3.2 (Table 6.1)). The PCR cycle parameters used were also optimised for each gene of interest (detailed in Section

3.3.4 and 6.3.2) but all followed a common protocol of a denaturation step followed by annealing, and then an elongation phase. These three stages are then repeated a number of times, dependent on the particular primers being used. The number of cycles used in the PCR protocols within this thesis varied from 26 to 40 cycles. A single subsequent extended elongation step was then carried out to complete the PCR cycles. Negative controls were used in each PCR reaction. One negative control involves the addition of water to the master mix in place of either a cDNA or DNA sample. Additionally, in Chapter 3 a no-reverse transcriptase (no-RT) negative was also included in each PCR experiment.

2.8.1 Gel Electrophoresis and Visualisation of PCR Products

After removal from the PCR machine each 25µl sample had 5µl of loading buffer added to it and was mixed thoroughly. Loading buffer consisted of glycerol 50% (Sigma-Aldrich, UK) and 0.5M EDTA pH8 0.04% (Sigma-Aldrich, UK) in distilled H_2O. This step was carried out in a separate area than that used to set up the PCR reactions. Gel electrophoresis was used to establish the presence and molecular weight of the PCR products. 2% agarose gels (Flowgen Bioscience Ltd, UK) containing ethidium bromide were poured into a mini-gel tank (BioRad Laboratories Ltd, UK) and allowed to set for at least 45 minutes before being covered with 1x TBE (not containing ethidium bromide) as a running buffer. The wells in the gel were loaded with 10µl of the PCR product (containing loading buffer) or ladder and run at 50V (powerpack - BioRad Laboratories Ltd, UK) for between 1-2 hours until the ladder and products were well separated. The ladder used for product size determination and, where required, quantification was the DNA molecular weight

marker XIV (Roche, Germany). Aliquots of this ladder were stored at -20°C avoiding repeated freeze/thawing. The PCR products were visualised with a UV transilluminator (BioRad Laboratories Ltd, UK) and images captured with an attached camera and computer system (Alpha Imaging System, Alpha Innotech Corporation, USA).

2.9 Confocal Imaging

To enable confocal images to be acquired oocytes were placed on Superfrost positively charged slides (VWR International Ltd, UK) and excess liquid removed to allow adherence to the glass. The oocytes were then mounted in 10µl of vectashield, stored at 4°C, (Vector Laboratories, USA) and covered with a glass slide (thickness 1, BDH, UK). Slides were stored at 4°C, wrapped in foil, before imaging for no more than 6 hours to limit any loss of fluorescent signal. Samples were observed using the Leica upright TCS-NT confocal microscope with Argon/Krypton laser (Leica Microsystems Ltd, UK). Either single oocyte images or Z-stacks, using the automated stage, were obtained using the Leica Imaging software (Leica Microsystems Ltd, UK). All images were taken using a dry x40 lens followed by x2 zoom, with a pinhole setting of 1 airy unit and constant photomultiplier tube (PMT) setting within each experiment.

Chapter 3

The effect of exogenous gonadotrophins on the expression level of genes involved in regulating DNA methylation

3.1 INTRODUCTION

Although the effects of oestrogens and androgens within the follicle have been the subject of extensive investigation, the full extent of the role they play remains to be elucidated. Previous results from this laboratory used an individual follicle culture system to investigate the effects of raised steroid levels on follicle development. Although there was no apparent effect on either follicular growth or rates of atresia, there was an impact on the developmental competence of the oocyte (refer to Section 1.15). After exposure to raised levels of an oestrogenic compound, DES, oocytes exhibited a reduced fertilisation rate during IVF. In contrast, exposure to raised androgen levels was found to improve the percentage of oocytes undergoing successful fertilisation (Figures 1.9A and 1.11A). This result raised the question of what the mechanism was by which raised oestrogen and androgen influenced the developmental competence of these oocytes.

The important role that genomic imprinting of germ cells plays on embryo development is well established. Severe disruptions to this mechanism are detrimental to subsequent development and in some cases even embryo lethal. Thus, the laying down of DNA methylation on the oocyte genome is a vital process of oocyte maturation. It is known that maternal imprints are laid down in growing, rather than quiescent, oocytes. Each imprinted gene becomes methylated at a gene-specific time, during primary to pre-ovulatory development (Kono *et al.*, 1996; Bao *et al.*, 2000; Obata & Kono, 2002; Lucifero *et al.*, 2004). It has previously been shown that some pre-implantation embryos, exposed to a period of time in culture, have altered expression of imprinted genes (Doherty *et al.*, 2000; Khosla *et al.*, 2001;

Mann *et al.*, 2004). It may be that during the period of epigenetic reprogramming in the early embryo the imprinted genes, which should maintain their imprints, are particularly vulnerable to changes in environment (Doherty *et al.*, 2000; Khosla *et al.*, 2001). In a similar way, the initial laying down of maternal imprints may be more susceptible to environmental factors during the relevant period of oocyte development. Perhaps then, it is not surprising that the environment that the oocyte is exposed to, as the maternal imprints are being acquired, has been found to alter imprinted gene expression. This is demonstrated by the altered methylation status of specific imprinted genes of oocytes after follicle culture, with the *Igf2r* locus losing methylation while *H19* has been shown to become hypermethylated during culture (Kerjean *et al.*, 2003).

Potentially, there are multiple factors which could contribute to errors in genomic imprinting occurring after oocytes or embryos have been exposed to culture conditions. There is no single conclusive piece of evidence which demonstrates that altered hormonal exposure reduces oocyte developmental competence, through alterations to the mechanisms involved in genomic imprinting. However, there is an accumulation of data which suggest that epigenetic changes, which occur during oocyte growth, are influenced by the hormonal environment. The percentage of embryos with impaired blastocyst development increases, both *in vitro* and *in vivo*, when they are derived from superovulated rather than naturally ovulated oocytes (Ertzeid & Storeng, 2001; Van der Auwera & D'Hooghe, 2001). The exogenous administration of superovulatory gonadotrophins results in alterations to the steroidal milieu that the oocyte is exposed to during growth and maturation (Kajta, 1998).

Therefore, the reduced developmental capacity of the subsequent embryos suggests that aberrant oocyte maturation occurs when the hormonal environment is altered. Children conceived with the aid of ARTs appear to have an increased incidence of normally rare imprinting disorders (Cox *et al.* 2002; Orstavik *et al.* 2003; DeBaun *et al.*, 2003; Maher *et al.* 2003; Gicquel *et al.* 2003). This may represent a correlation between altered oocyte and embryo environment, with errors in the mechanisms of genomic imprinting. Of particular relevance may be a recent study examining the incidence of BWS after ART. There was not found to be any correlation between the cause of parental infertility, type of ART or culture medium used and the conceptions resulting in the birth of BWS children (Chang *et al.*, 2005). The only common factor between all the cases of BWS after ART examined in this study was found to be ovarian stimulation (Chang *et al.*, 2005). The small sample size means no conclusive role of ovarian stimulation in the development of BWS in subsequent offspring can be proved. However, this finding supports a potential impact of raised gonadotrophin and steroid levels on the epigenetic stability of the oocyte. Another investigation has found that embryos exposed to altered culture conditions can have aberrant DNA methylation levels (Shi & Haaf, 2002). However, the use of superovulation prior to either *in vivo* or *in vitro* fertilisation means it has not been possible to determine whether the impaired DNA methylation originates in the oocyte or occurs during early embryo development (Shi & Haaf, 2002). The possibility that superovulated oocytes have altered DNA methylation in response to the altered steroid exposure is unresolved, but could account for the observed reduction in developmental competence.

Utilising nuclear transfer, to investigate the developmental potential of chromatin from different stage oocytes, demonstrated that those isolated from adults had acquired the necessary epigenetic modifications earlier in development than those from juvenile mice (Bao *et al.*, 2000). One possibility is that oocytes from adult mice have been exposed to a different gonadotrophin and steroidal environment than those from juvenile animals. This alteration in hormonal exposure may account for the time difference in acquiring epigenetic maturity (Bao *et al.*, 2000).

A correlation between hormonal environment, including altered steroid levels, and impaired developmental competence has been repeatedly demonstrated (Moor *et al.*, 1980; Andersen, 1993; Zelinksi-Wooten *et al.*, 1993; Silva & Knight, 2000; Xia & Younglai, 2000). Additionally, the requirement for correct genomic imprinting in normal development is now evident. Investigations into the effect of steroids on genomic imprinting have been restricted due to the delayed and sometimes minor impact on development. Many epigenetic changes are not evident during early development, while some, such as those leading to the development of cancer, may not be seen until adult life (Mizuno *et al.*, 2001; Szyf, 2003). This delay in any observable phenotype is also demonstrated by several strains of KO mice, such as animals lacking *Mbd2* or *Dnmt3l* (Hendrich *et al.*, 2001; Bourc'his *et al.*, 2001). This has complicated the issue of identifying both the time at which epigenetic mutations occur, and the factors which cause them.

3.1.1 *Dnmt* and *Mbd* genes in the Ovary

One cause of inappropriate DNA methylation is a change in the transcription or translation of the *Dnmt* genes. The enzymes encoded for by this family of genes are responsible for de novo and maintenance methylation, as previously discussed in section 1.12. However, the influence of any single Dnmt is complicated by the high level of co-operation that has been identified as existing between several enzymes in this family (Kim *et al.*, 2002; Liang *et al.*, 2002). The expression level and localisation of each Dnmt in the ovary is closely controlled, as both inappropriate levels and timing of DNA methylation can have detrimental effects on development (Kaneda *et al.*, 2004; Biniszkiewicz *et al.*, 2002).

Dnmt1 splice variants act as maintenance methylases *in vivo* although, *in vitro* they also have de novo methylase activity (Howell *et al.*, 2001). As this study is focused on the *Dnmts* present in the ovary only *Dnmt1s* and *1o* splice variants will be considered. Although both *Dnmt1s* and *Dnmt1o* mRNA transcripts are present in the oocyte, there is no translation of Dnmt1s and only the Dnmt1o protein is found (Ratnam *et al.*, 2002, Figure 5.1A). Initial studies analysing the *Dnmt1* transcripts in both embryos and the testes have begun to determine factors that influence this gene's expression. Pre-implantation mouse embryos exposed to a period of time in culture have a significant increase in *Dnmt1* expression when compared to *in vivo* obtained embryos (Wang *et al.*, 2005). In the testes of hypophysectomised rats there is no change to *Dnmt1s* levels, either in the absence of androgens or after exogenous testosterone administration, suggesting that this gene is not hormonally regulated in the male gonad (Jue *et al.*, 1995). However, no previous study has been carried out

to determine whether there is hormonal regulation of any of the *Dnmt* genes in the ovary. As previously discussed in section 1.12, Dnmt2 has only recently had a role in active methylation attributed to it. The binding ability of this enzyme was initially overlooked, as it has a highly specific binding pattern in mammals.

Dnmt3a, 3b and 3l are all expressed in the growing oocyte. As previously discussed in Section 1.13.2 the use of transgenic mice has allowed preliminary study of the role of each of these Dnmt proteins in the oocyte. In the absence of Dnmt3b normal maternal imprints are present, however, use of a germ cell specific conditional *Dnmt3a*$^{-/-}$ mouse line reveals that Dnmt3a is required for maternal imprinting in the oocyte and methylation at IAPs (Kaneda *et al.*, 2004). Although Dnmt3l has no active methyltransferase activity a vital regulatory role in genomic imprinting is demonstrated by the phenotype *Dnmt3l*$^{-/-}$ mice. The oocytes of female *Dnmt3l*$^{-/-}$ mice with disrupted *Dnmt3l* alleles do not contain the normal maternal imprints, resulting in an embryo lethal maternal effect (Bourc'his *et al.*, 2001).

It has been established that oocyte quality can be impaired by sub-optimal environmental conditions during oocyte maturation and that the effects of this may only become evident during embryonic or even more advanced stages of development. However, the full range of factors and the mechanisms by which they influence oocyte maturation are not yet known. Superovulation is a regularly used technique both in medical and research settings which results in alteration of the hormonal environment during oocyte maturation by the administration of exogenous gonadotrophins. However, there are some reports of impaired developmental competence of oocytes after superovulation which raises questions as to the role of

raised gonadotrophins and steroid levels on oocyte maturation. Additionally, the recent observation that children born after ART, where oocytes will have been obtained after ovarian stimulation, appear to have an increased incidence of imprinting disorders strengthens the need for an increased understanding of the effects of raised hormone levels on the oocyte. Potentially, the increased levels of gonadotrophins and steroids could be having an effect on the process of genomic imprinting within the oocyte during maturation. The laying down of maternal imprints requires the activity of Dnmt proteins, thus any alterations to the expression of these genes could impair the initiation of normal maternal DNA methylation. The subsequent repression of methylated genes requires the presence of bound Mbd proteins, thus changes to the expression of the genes involved in genomic imprinting could cause reduced developmental competence of oocytes and imprinting disorders in later life. This chapter aims to investigate the possibility that the administration of exogenous gonadotrophins could have an effect on *Dnmt* or *Mbd* gene expression in the oocyte. Initial work to optimise the protocols required to analyse expression of these genes will be carried out on whole ovary as these samples will provide larger levels of RNA to work with. Subsequently, these established protocols can be used to analyse the gene expression in isolated oocytes to identify any effect of high levels of exogenous gonadotrophins on the expression of *Dnmt* or *Mbd* genes.

3.2 AIM

The aim of this study was to determine whether the administration of exogenous gonadotrophins, is associated with alteration of ovarian *Dnmt* and *Mbd* gene expression levels.

3.3.1 Experimental Protocol

Three F1 females were allocated to each of the treatment groups at three weeks of age, as detailed in Table 3.1. Pregnant mare serum gonadotrophin (PMSG) (Intervet UK Limited, UK), recombinant human (rh) LH (Serono, Switzerland) and human chorionic gonadotrophin (hCG) (Intervet UK Limited, UK) were made up in saline to a concentration of 5 IU/100µl and injected subcutaneously. The two injections were given 48 hours apart. Animals were killed by cervical dislocation 5 hours after administration of the second injection, prior to ovulation occurring. The ovaries were removed as described in Section 2.4 and immediately snap frozen in liquid nitrogen. The intact frozen ovaries were then transferred to a -80°C freezer where they were stored until required for RNA extraction. Two runs of this experimental protocol were carried out.

3.3.2 RNA Extraction

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, UK) according to the manufacturers instructions provided with the kit for the isolation of Total RNA from animal tissues. An overview of this protocol is as follows. Ovarian tissue samples were initially disrupted in lysis buffer from frozen using a sterile test-tube pestle and mortar system. All equipment was RNase-free. To ensure a complete homogenisation of the small ovarian samples with the lysis buffer, the tissue sample was passed through a sterile insulin syringe (Sherwood, UK) 6 times. Homogenates were centrifuged for 3 minutes at maximum speed and the subsequent supernatant transferred to a fresh microcentrifuge tube. 1 volume of 70% ETOH was added to

this lysate and mixed thoroughly. This mixture was then transferred into a RNeasy mini-column (provided with the kit) placed in a collection tube before centrifugation at >10,000 rpm for 15 seconds. The flow through was then discarded and additional Buffer RPE from the kit added onto the column. The centrifugation step was repeated and the collection tube contents again discarded. A further 500µl of buffer RPE was pipetted onto the column followed by a 2 minute spin at >10,000 rpm. After the column has been placed into a fresh collection tube a further centrifugation step was carried out to ensure no carry over of buffer. The final elution step involved placing the column into a fresh tube before adding 50µl of RNase-free water to the column and carrying out a final spin for 1 minute at >10,000rpm. Once extracted the ovarian RNA samples from each treatment group were pooled. Samples were then frozen and stored at -70°C until required for cDNA synthesis.

3.3.3 cDNA synthesis from total RNA

2µl of total RNA had 1µl of Random Primers (200ng/µl, Promega, UK), 1µl dNTP Mix (10mM of dATP, dCTP, dGTP and dTTP, Promega, UK) and 8µl water added to it before being heated at 65°C for 5 minutes followed by cooling on ice for a further 2 minutes. After this 4µl x5 MMLV-RT reaction buffer (Promega, UK), 2µl DTT (0.1M, Promega) and 1µl RNase inhibitor (Sigma-Aldrich, UK) were added to the mixture which was left at room temperature for 2 minutes. At this stage 1µl M-MLV-RT (200U, Promega, UK) was added followed by a further 10 minutes at room temperature and then an incubation of 50 minutes at 37°C. The final step was deactivation of the enzyme by heating at 70°C for 15 minutes. The no-RT control

was produced by the same protocol but with the exception of the M-MLV-RT being replaced by sterile water. All tubes were then cooled and stored at -20°C.

Table 3.1:- Injection protocol for each treatment group. PMSG – Pregnant Mares Serum Gonadotrophin; hCG – Human Chorionic Gonadotrophin; LH – Luteinising Hormone

TREATMENT	INJECTION 1	INJECTION 2
Treatment 1	Saline	Saline
Treatment 2	PMSG	Saline
Treatment 3	PMSG	hCG
Treatment 4	PMSG/LH	Saline
Treatment 5	PMSG/LH	hCG

3.3.4 RT-PCR

The master mix for PCR containing x10 buffer (Promega, UK), magnesium (Promega, UK), dNTPs (Promega, UK)), and platinum *Taq* polymerase (Invitrogen, UK) had the appropriate PCR primers (Eurogentec, UK) added as detailed in Table 3.2. The concentration of each constituent and the PCR programme were optimised for each gene of interest as shown in Table 3.3 A and B and Table 3.4. RT-PCR, gel electrophoresis and visualisation of the products was carried out as described in Section 2.8 and 2.8.1. PCR amplification of samples of interest, an H_2O blank and no-RT negative control were carried out in parallel in all experiments.

Table 3.2:- Table detailing the RT-PCR primers used for each gene of interest. **A)** contains the *Dnmt* gene primer details while **B)** contains those for *Mbd*, *MeCP2* and *cyclophilin.*

A

Gene	F/R	Primers	Accession Number	Primer Position	Intron Spanning	Reference
Dnmt1s	F	GGGTCTCGTTCAGAGCTG	The details of these primers are published in the reference given			Mertineit *et al.*, 1998
	R	GCAGGAATTCATGCAGTAAG				
Dnmt1o	F	GGTTGATTGAGGGTCATT	The details of these primers are published in the reference given			Mertineit *et al.*, 1998
	R	GCAGGAATTCATGCAGTAAG				
Dnmt3a	F	TCCCCCACACCAGCTCTCC	The details of these primers are published in the reference given			Chen *et al.*, 2002
	R	TCCCGGGGCCGACTGCGA				
Dnmt3a2	F	TCCCCCACACCAGCTCTCC	The details of these primers are published in the reference given			Chen *et al.*, 2002
	R	AGGGGCTGCACCTGGCCTT				
Dnmt3b	F	GCTCAGACCTGGCTGCTTAG	Dnmt3b1 AF68626 Dnmt3b2 AF068627 Dnmt3b3 AF068628	3044	✓	Ian Simpson
	R	AGGGAAAAGCCCTAAAGGTG		3532		
Dnmt3l	F	ACATCTGCCTCTGCTGTGGAACTC	The details of these primers are published in the reference given			Aapola *et al.*, 2001
	R	TCCCTCTTGATCATGGAAGGCCTT				

F=forward primer. R=Reverse primer

Table 3.2 continued:- Table detailing the RT-PCR primers used for each gene of interest. **A)** contains the *Dnmt* gene primer details while **B)** contains those for *Mbd*, *MeCP2* and *cyclophilin.*

B

Gene	F/R	Primers	Accession Number	Primer Position	Intron Spanning?	Reference
Mbd1	F	AGCGCCAGTGGAGGTGTTTG	AF072240	984-1003	✓	Donncha Dunican
	R	GCTCGTCCTCGTCCTCGTCT		1186-1205		
Mbd2	F	CCTGGGAAATGCTGTTGACC	AF072249	680-699	✓	Donncha Dunican
	R	GGACCGACTCCTTGAAGACC		1014 -1033		
Mbd3	F	AGCCTTCATGGTGACAGATG	AF072248	896-915	✓	Donncha Dunican
	R	GACGGCTGCCTCCTACAGGT		1175-1194		
Mbd4	F	CGTGTGGATGGGAAAGAGTT	AF072249	369-388	✓	Designed using Primer3
	R	GGAAGTCAGAGCTGCCAAAC		585-604		
MeCP2	F	CCCCCAAACAGCGGCGCTCC	AF158181	421-440	✓	Donncha Dunican
	R	GCTCCCTCTCCCAGTTACCGTG		654-674		
Cyclophilin	F	CCAGGGTGGTGACTTTACAC	NM_008907	227-246	✓	Designed using Primer3
	R	CGGAAATGGTGATCTTCTTG		500-519		

F=forward primer. R=Reverse primer

Table 3.3:- Tables showing the RT-PCR protocols for each *Dnmt* gene (**A**) and each *Mbd*, *MeCP2* and *cyclophilin* gene (**B**).

A	***Dnmt1s***	***Dnmt1o***	***Dnmt3a***	***Dnmt3a2***	***Dnmt3b***	***Dnmt3l***
	Final Concentrations					
10X PCR Buffer	1X	1X	1X	1X	1X	1X
MgCl2	1.2mM	1.2mM	1.2mM	1.2mM	1.2mM	0.6mM
dNTP mixture (5mM each)	0.2mM each	0.2mM each	0.2mM each	0.2mM each	0.2mM each	0.05mM each
sense primer (100µM)	0.8µM	0.8µM	0.8µM	0.8µM	0.8µM	0.8µM
anti-sense primer (100µM)	0.8µM	0.8µM	0.8µM	0.8µM	0.8µM	0.8µM
Platinum *Taq* Polymerase (5U/µl)	1U	1U	1U	1U	1U	0.65U
Autoclaved distilled H_2O	Make total volume up to 25µl					
Cycle Number	26	26	26	32	30	40
Anneal temp	55	55	61	61	55	64
Programme	A	A	B	B	B	C

B	***Mbd1***	***Mbd2***	***Mbd3***	***Mbd4***	***MeCP2***	***cyclo***
	Final Concentrations					
10X PCR Buffer	1X	1X	1X	1X	1X	1X
MgCl2	0.24mM	1.2mM	0.24mM	1.2mM	1.2mM	0.24mM
dNTP mixture (5mM each)	0.05mM each	0.2mM each	0.05mM each	0.2mM each	0.2mM each	0.05mM each
sense primer (100µM)	0.8µM	0.8µM	0.8µM	0.8µM	0.8µM	0.8µM
anti-sense primer (100µM)	0.8µM	0.8µM	0.8µM	0.8µM	0.8µM	0.8µM
Platinum *Taq* Polymerase (5U/µl)	1U	1U	1U	1U	1U	1U
Autoclaved distilled H_2O	Make total volume up to 25µl					
Cycle Number	26	26	26	35	35	26
Anneal temp	58	55	55	55	55	55
Programme	B	B	B	B	B	B

Table 3.4:- Table listing the RT-PCR cycle parameters for the genes specified in Table 3.3. To determine the value of X refer to appropriate gene in Table 3.3.

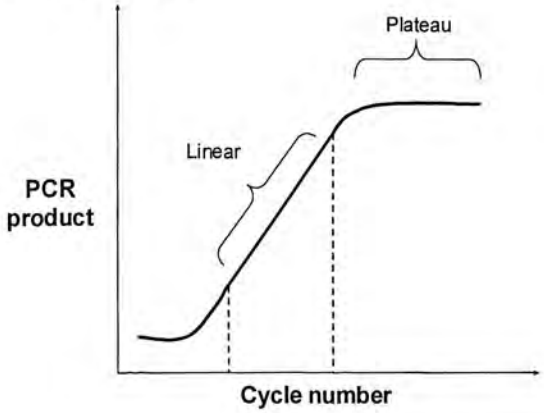
	PCR Programme A	PCR Programme B	PCR Programme C
Denaturation	94°C for 10 sec	95°C for 1 min	94°C for 3 mins
Annealing	55°C for 10 sec	X°C for 1 min	94°C for 20 sec
Elongation	72°C for 40 sec	72°C for 1 min	64°C for 30 sec
Number of cycles	35	X	X
Extended Elongation	72°C for 2 min	72°C for 7 min	72°C for 1 min

3.3.5 Semi-Quantitative Analysis

3.3.5.1 Linear Range

To allow the amount of PCR product present relative to the housekeeping gene, cyclophilin, to be evaluated the PCR reaction for all genes had to be within the linear range. That is the kinetics of the reaction must not have reached the plateau caused by a component of the master mix having become a rate limiting factor (Figure 3.1). The linear range of all the genes of interest and the housekeeping gene, *cyclophilin*, were established by comparing the amount of product synthesised after different numbers of PCR cycles.

Figure 3.1:- The kinetics of PCR

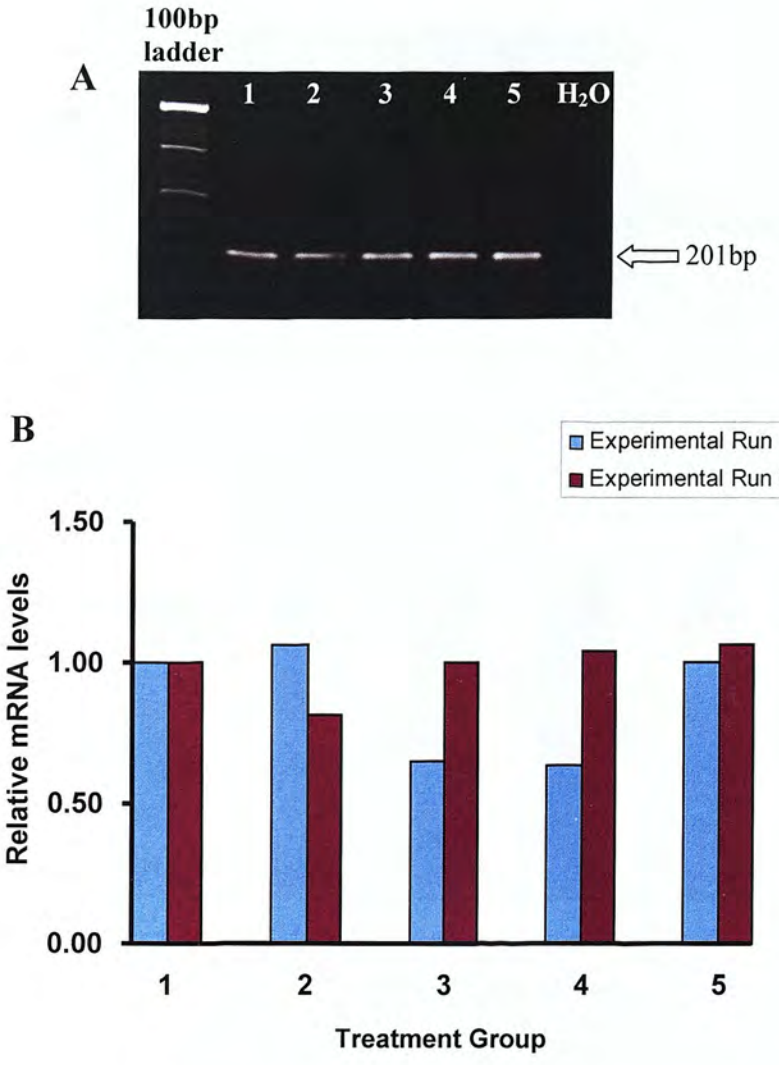


3.3.5.2 Quantitation

The yield of cDNA was determined by analysing the density of the housekeeping gene PCR product present after 26 cycles. Densitometry analysis was carried out using the Alpha Imaging System Software (Alpha Innotech Corporation, USA). The volume of cDNA used in the subsequent RT-PCRs was altered such that samples from all treatment groups gave the same level of *cyclophilin* PCR product. The densitometry facility on the gel imaging software was used to analyse the density of each gene of interest PCR product band. The band from each treatment group was then normalised to the Group 1 control which was allocated a value of 1.

3.4 RESULTS

RT-PCR was carried out for each *Dnmt* and *Mbd* gene and the results were quantified using comparative densitometry. The RT-PCR gels showing the PCR products for each treatment from one run of this experiment are shown for each gene (Figures 3.2-3.11). In all cases H_2O and no-RT negatives were also loaded onto gels and visualised after electrophoresis. All of these samples gave the expected negative result. The normalised densitometry results for each of the two experimental runs were both plotted on a single graph for each gene (Figures 3.2-3.11). For some genes, such as *Dnmt1o* (Figure 3.3) and *MeCP2* (Figure 3.11) consistent results were obtained from the two experimental runs. However, when compared to the saline controls, there was no change in the expression levels of these genes after treatment. In other cases, such as *Dnmt3a* (Figure 3.4) and *Dnmt3b* (Figure 3.6) there appeared to be differences in expression level with gonadotrophin treatment. However, no consistent pattern was seen across the two experimental repeats.



Key

1. Saline/Saline	4. PMSG + LH/Saline
2. PMSG/Saline	5. PMSG + LH/hCG
3. PMSG/hCG	

Figure 3.2:- Semi-quantitative RT-PCR for *Dnmt1s*. **A)** RT-PCR gel image, lane numbers represent treatment groups and the arrow represents product size. **B)** Graph of densitometry results normalised to the Group 1 value, allocated a value of 1, within each PCR run. PMSG – Pregnant Mare Serum Gonadotrophin; hCG – human chorionic gonadotrophin; LH – Luteinising hormone. Three mice were used for each treatment group and the ovarian mRNA pooled. Two experimental replicates were carried out.

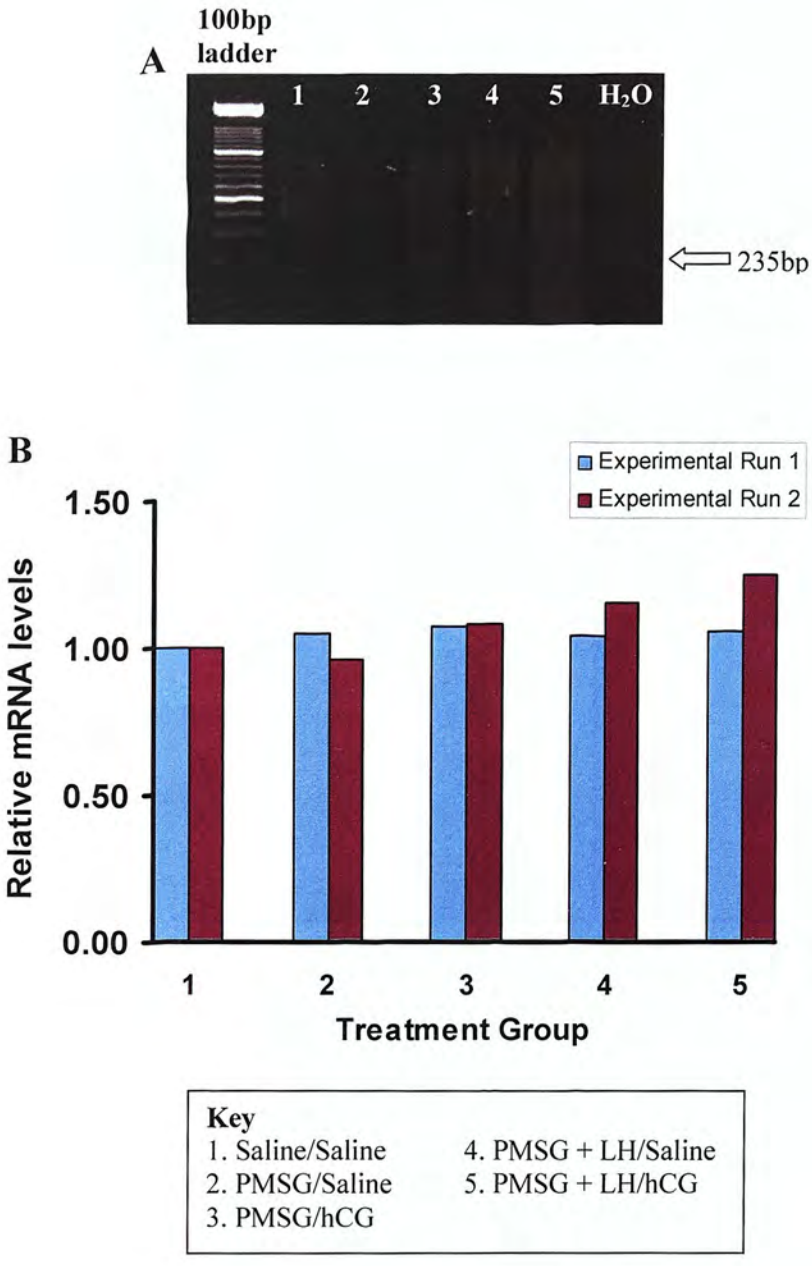


Figure 3.3:- Semi-quantitative RT-PCR for *Dnmt1o* **A)** RT-PCR gel image, lane numbers represent treatment groups and the arrow represents product size. **B)** Graph of densitometry results normalised to the Group 1 value, allocated a value of 1, within each PCR run. PMSG – Pregnant Mare Serum Gonadotrophin; hCG – human chorionic gonadotrophin; LH – Luteinising hormone. Three mice were used for each treatment group and the ovarian mRNA pooled. Two experimental replicates were carried out.

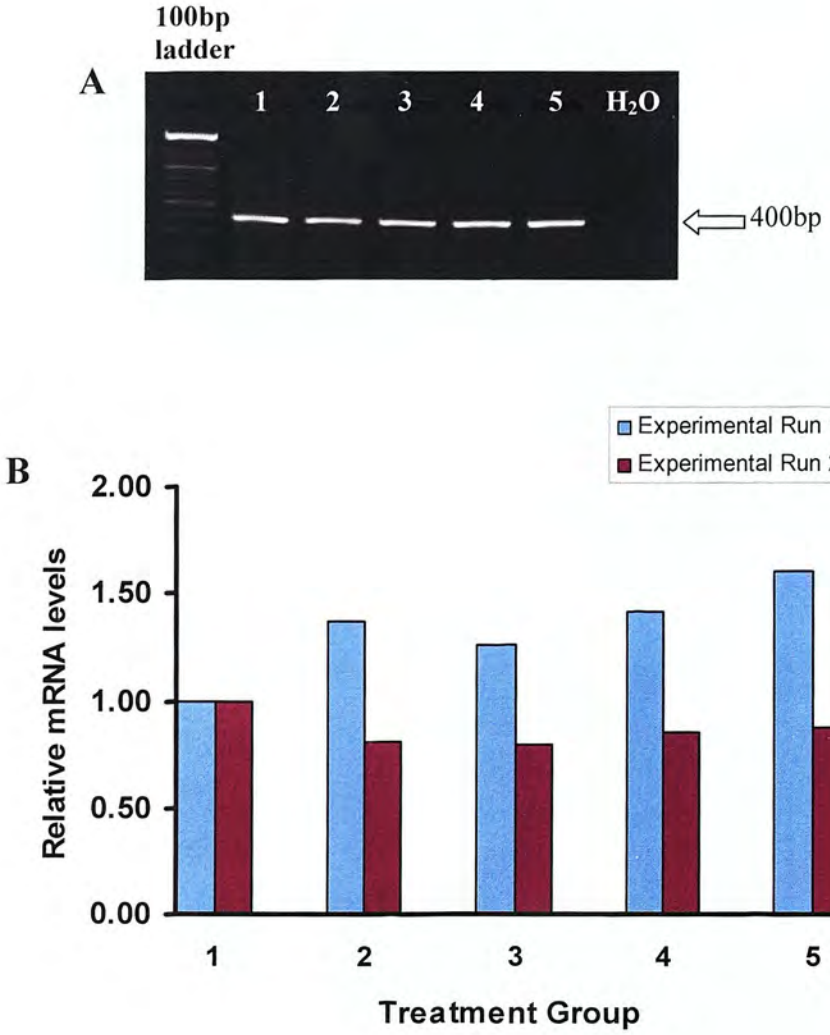


Figure 3.4:- Semi-quantitative RT-PCR for *Dnmt3a* **A)** RT-PCR gel image, lane numbers represent treatment groups and the arrow represents product size. **B)** Graph of densitometry results normalised to the Group 1 value, allocated a value of 1, within each PCR run. PMSG – Pregnant Mare Serum Gonadotrophin; hCG – human chorionic gonadotrophin; LH – Luteinising hormone. Three mice were used for each treatment group and the ovarian mRNA pooled. Two experimental replicates were carried out.

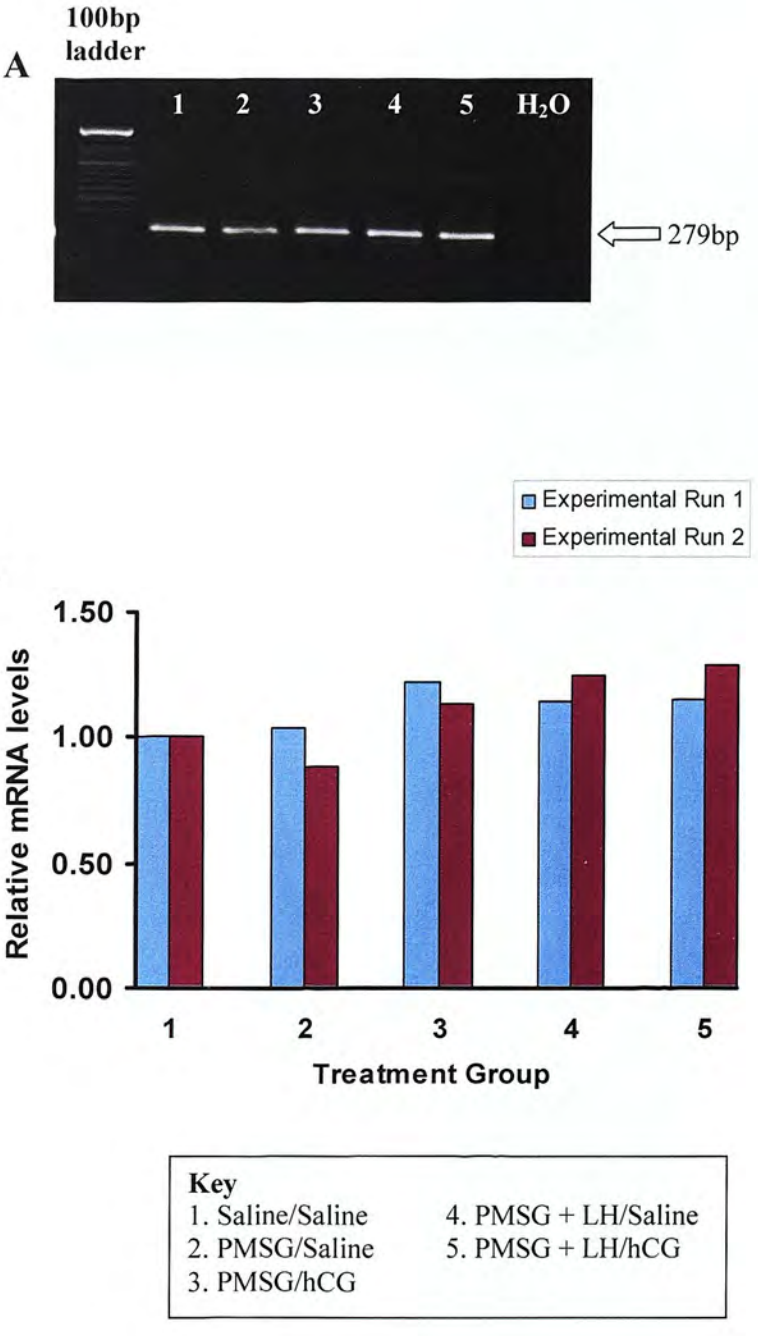
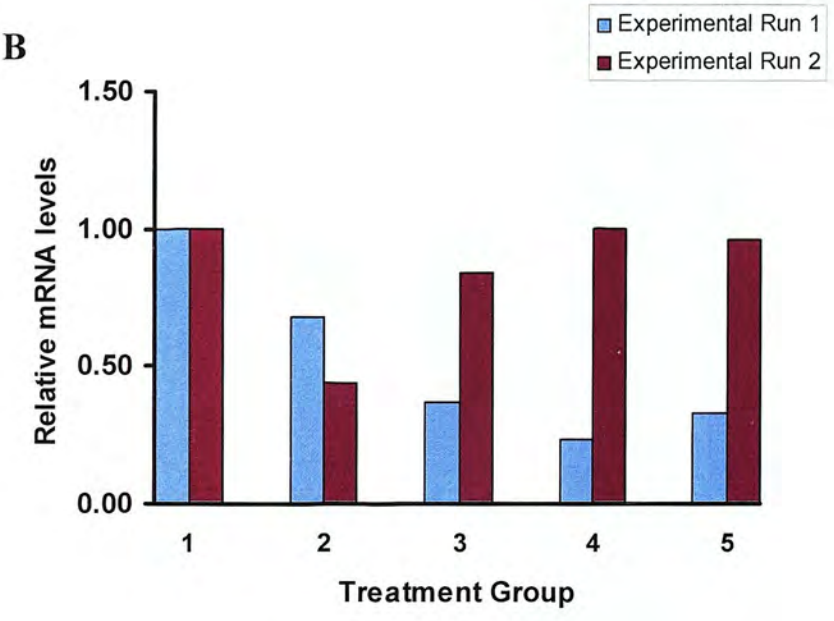


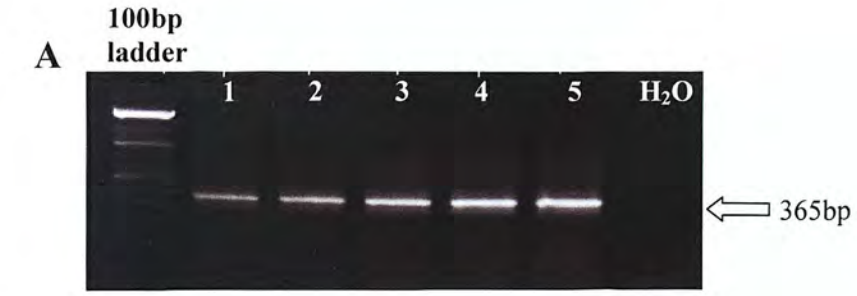
Figure 3.5:- Semi-quantitative RT-PCR for *Dnmt3a2*. **A)** RT-PCR gel image, lane numbers represent treatment groups and the arrow represents product size. **B)** Graph of densitometry results normalised to the Group 1 value, allocated a value of 1, within each PCR run. PMSG – Pregnant Mare Serum Gonadotrophin; hCG – human chorionic gonadotrophin; LH – Luteinising hormone. Three mice were used for each treatment group and the ovarian mRNA pooled. Two experimental replicates were carried out.

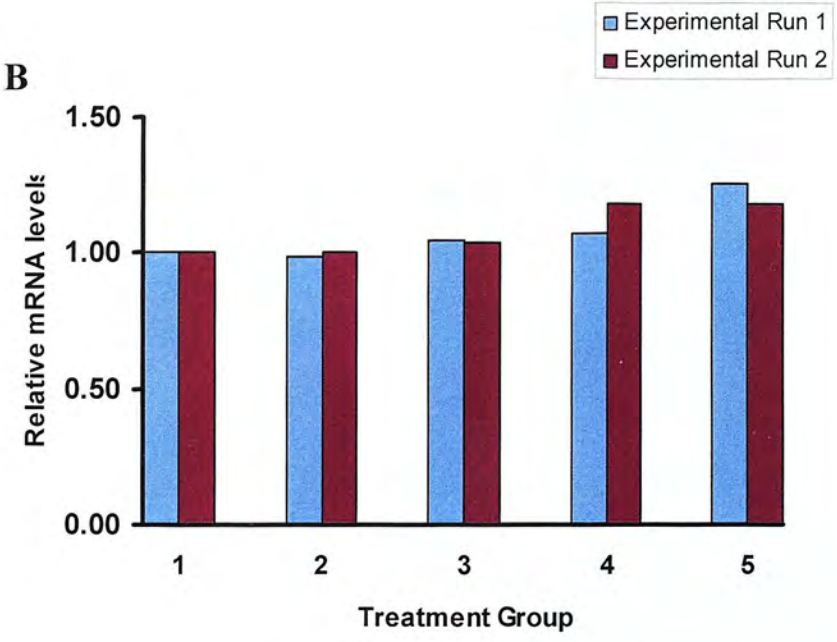


Key

1. Saline/Saline	4. PMSG + LH/Saline
2. PMSG/Saline	5. PMSG + LH/hCG
3. PMSG/hCG	

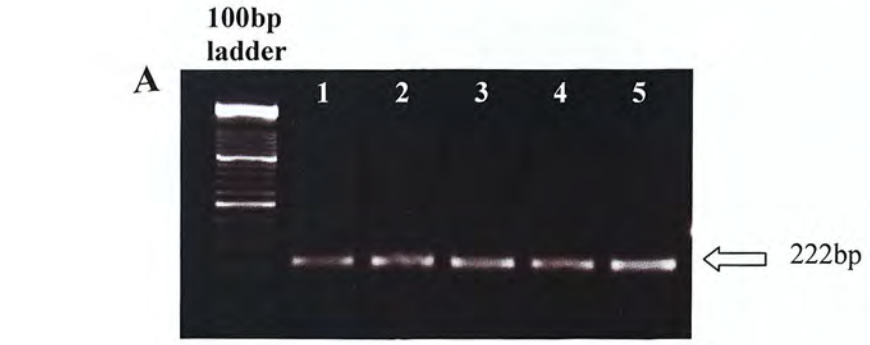
Figure 3.6:- Semi-quantitative RT-PCR for *Dnmt3b* **A)** RT-PCR gel image, lane numbers represent treatment groups and the arrow represents product size. **B)** Graph of densitometry results normalised to the Group 1 value, allocated a value of 1, within each PCR run. PMSG – Pregnant Mare Serum Gonadotrophin; hCG – human chorionic gonadotrophin; LH – Luteinising hormone. Three mice were used for each treatment group and the ovarian mRNA pooled. Two experimental replicates were carried out.

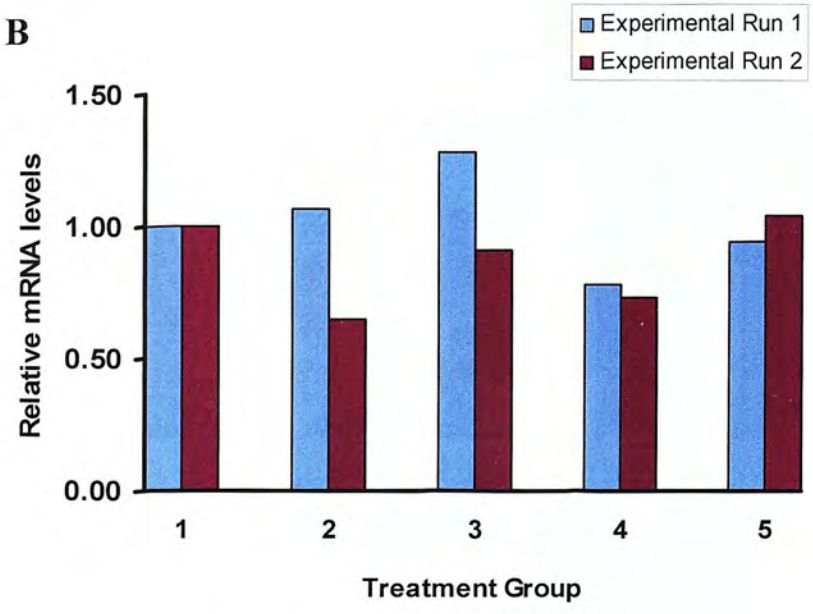




Key	
1. Saline/Saline	4. PMSG + LH/Saline
2. PMSG/Saline	5. PMSG + LH/hCG
3. PMSG/hCG	

Figure 3.7:- Semi-quantitative RT-PCR for *Dnmt3l* **A)** RT-PCR gel image, lane numbers represent treatment groups and the arrow represents product size. **B)** Graph of densitometry results normalised to the Group 1 value, allocated a value of 1, within each PCR run. PMSG – Pregnant Mare Serum Gonadotrophin; hCG – human chorionic gonadotrophin; LH – Luteinising hormone. Three mice were used for each treatment group and the ovarian mRNA pooled. Two experimental replicates were carried out.





Key

1. Saline/Saline	4. PMSG + LH/Saline
2. PMSG/Saline	5. PMSG + LH/hCG
3. PMSG/hCG	

Figure 3.8:- Semi-quantitative RT-PCR for *Mbd1* **A)** RT-PCR gel image, lane numbers represent treatment groups and the arrow represents product size. **B)** Graph of densitometry results normalised to the Group 1 value, allocated a value of 1, within each PCR run. PMSG – Pregnant Mare Serum Gonadotrophin; hCG – human chorionic gonadotrophin; LH – Luteinising hormone. Three mice were used for each treatment group and the ovarian mRNA pooled. Two experimental replicates were carried out.

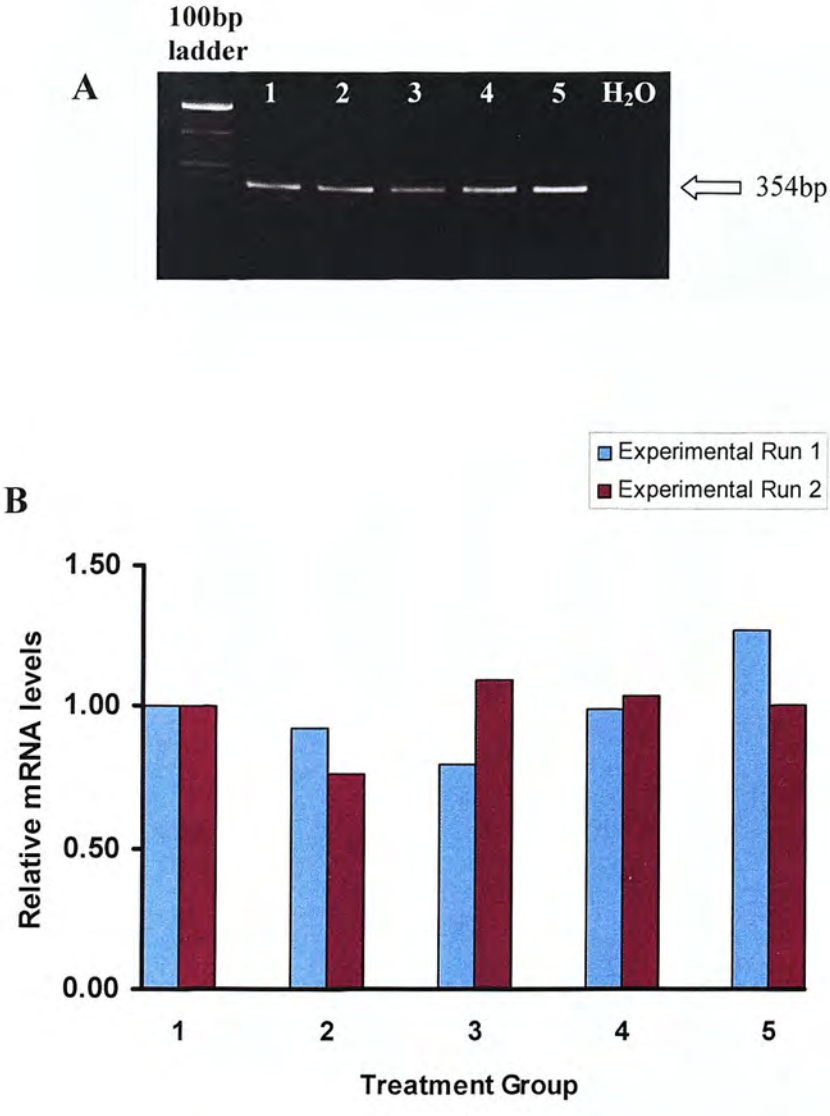


Figure 3.9:- Semi-quantitative RT-PCR for *Mbd2* **A)** RT-PCR gel image, lane numbers represent treatment groups and the arrow represents product size. **B)** Graph of densitometry results normalised to the Group 1 value, allocated a value of 1, within each PCR run. PMSG – Pregnant Mare Serum Gonadotrophin; hCG – human chorionic gonadotrophin; LH – Luteinising hormone. Three mice were used for each treatment group and the ovarian mRNA pooled. Two experimental replicates were carried out.

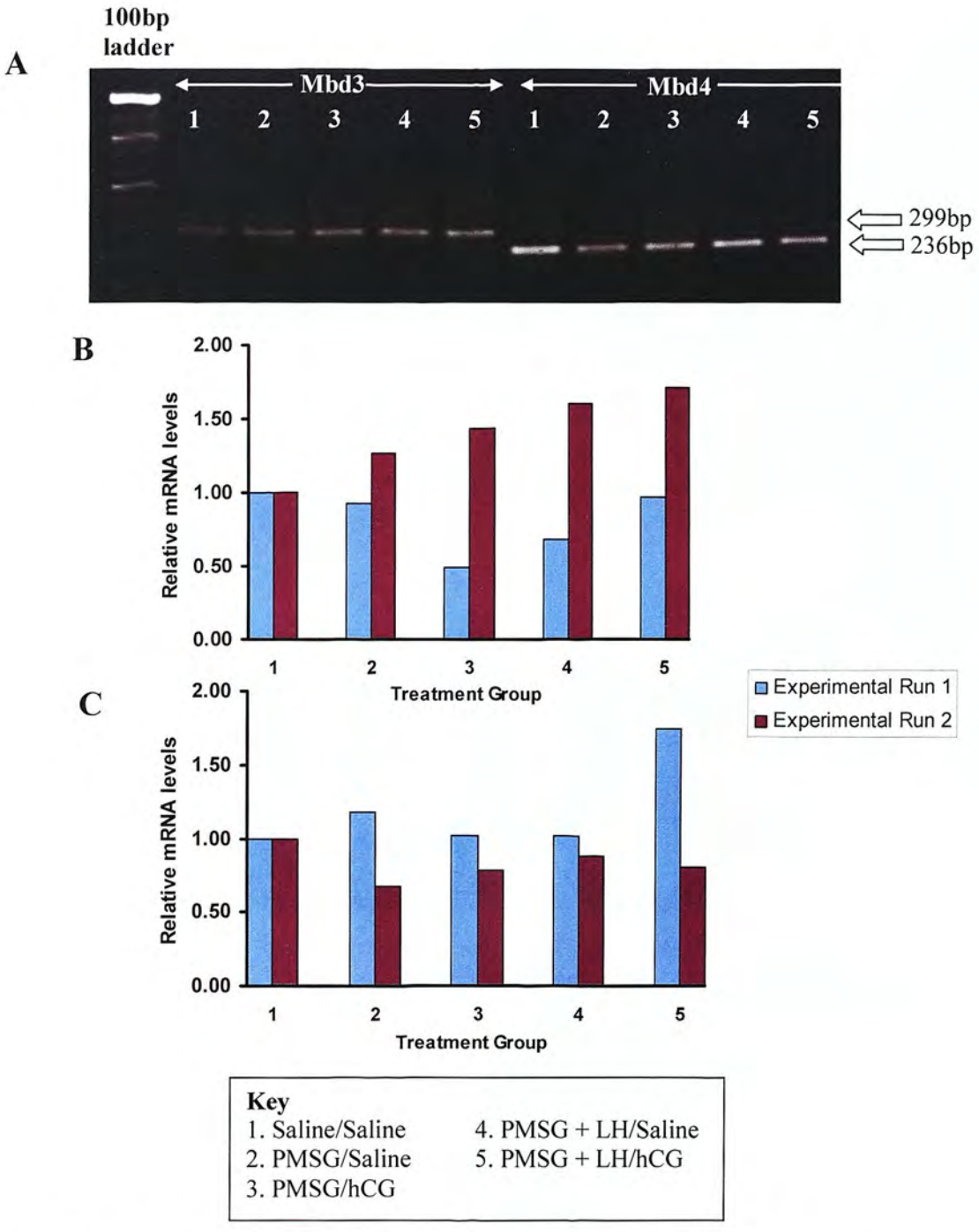
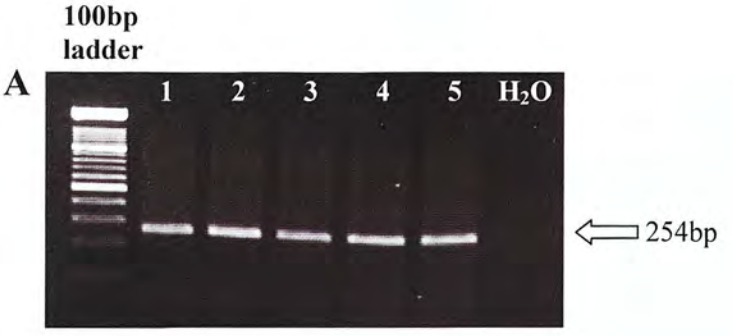
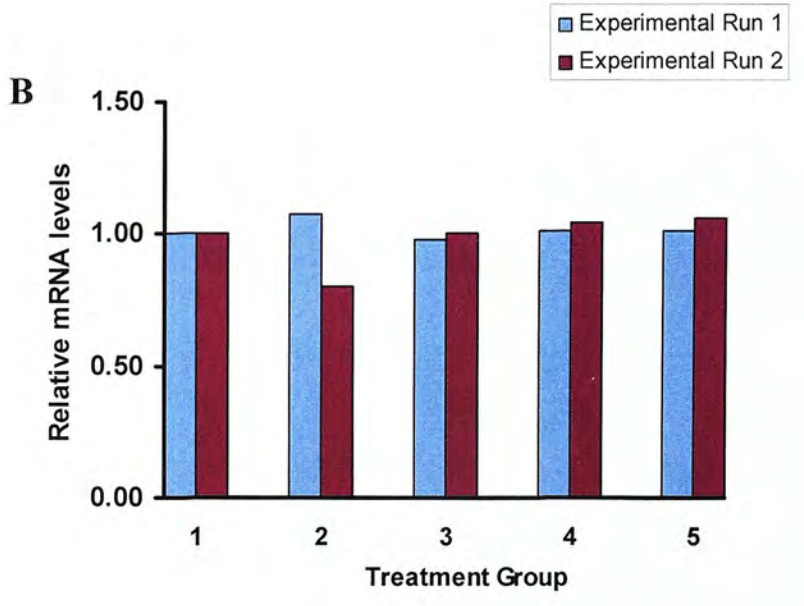


Figure 3.10:- Semi-quantitative RT-PCR for *Mbd3* and *Mbd4* **A)** RT-PCR gel image, lane numbers represent treatment groups and the arrow represents product size. **B)** *Mbd3* - Graph of densitometry results normalised to the Group 1 value, allocated a value of 1, within each PCR run. C) *Mbd4* – Graph of normalised densitometry results. PMSG – Pregnant Mare Serum Gonadotrophin; hCG – human chorionic gonadotrophin; LH – Luteinising hormone. Three mice were used for each treatment group and the ovarian mRNA pooled. Two experimental replicates were carried out.





Key	
1. Saline/Saline	4. PMSG + LH/Saline
2. PMSG/Saline	5. PMSG + LH/hCG
3. PMSG/hCG	

Figure 3.11:- Semi-quantitative RT-PCR for *MeCP2* **A)** RT-PCR gel image, lane numbers represent treatment groups and the arrow represents product size. **B)** Graph of densitometry results normalised to the Group 1 value, allocated a value of 1, within each PCR run. PMSG – Pregnant Mare Serum Gonadotrophin; hCG – human chorionic gonadotrophin; LH – Luteinising hormone. Three mice were used for each treatment group and the ovarian mRNA pooled. Two experimental replicates were carried out.

3.5 DISCUSSION

The initial aim of this study was to investigate alterations to gene expression levels in the oocyte. The start point in this experiment was to investigate any ovary wide changes in gene expression levels seen after the administration of exogenous gonadotrophins. Whole ovary was used as this would enable high levels of RNA to be extracted and allow optimisation of both the RT-PCR protocols for each *Dnmt* and *Mbd* gene and the quantification techniques. All of these genes investigated in this study, with the exception of Mbd1, Mbd4 and MeCP2, have previously been shown to be expressed in the postnatal mouse ovary (Mertineit *et al.*, 1998; Aapola *et al.*, 2001; Kantor *et al.*, 2003; Huntriss *et al.*, 2004; Lees-Murdock *et al.*, 2005; Vassena *et al.*, 2005).

However, even using whole ovary RNA there were problems with both optimisation and quantification which required extensive alterations to the protocol to resolve. As a result of these limitations of the technique the experimental samples analysed were limited to whole ovary and the intended extension of this analysis into follicles or oocytes alone was not carried out since very low RNA levels would be extracted from this material, adding yet further difficulties to the analysis.

When considering the ovarian samples obtained there was very little correlation between the PCR results obtained from each of the two experimental runs. As a result, no further runs of this experiment were carried out. This lack of further experimental runs precluded the use of statistical analysis on the data. Although, it

may be the case that the steroid hormones do not affect *Dnmt* or *Mbd* gene expression in any of the ovarian cell types, the methodology used in this study would not be sensitive enough to determine any subtle alteration in expression level. When the relative numbers of each cell type within the ovary are considered the proportion of mRNA that will have originated from the oocytes is small in comparison to that from the somatic cells. In addition, the presence of mRNA from all ovarian cells within each sample means the possibility of the exogenous gonadotrophins having different effects on each cell type could not be assessed. It is possible that a stimulus causing an up-regulation of gene expression in one cell type actually results in lowered gene expression in another. Thus, any subtle changes to gene expression levels in the oocyte would be masked. Additionally, it must be considered that several isoforms of the Dnmts exist, which would not have been individually detected by the primers used in this study. Isoform specific primers were used for Dnmt3a and Dnmt3a2 but this was not the case for Dnmt3b. The current study utilised pan-Dnmt3b primers so any change in transcription level of individual isoforms would not have been detected (Chen *et al.*, 2005).

There is also the possibility that either translation or activity of the proteins are altered by an altered hormonal environment. A family of avian methylated DNA binding proteins (MDBPs) normally bind to methylated DNA but this binding activity is reduced in the presence of oestradiol (Jost *et al.*, 1991). This is thought to be a control mechanism to ensure the normal expression of vitellogenin, a vital egg component, in the female liver. Oestradiol causes a partial dephosphorylation of MDBP-2 reducing its binding efficiency (Bruhat & Jost, 1995; 1996). The murine

Dnmt1 has been found to be post-translationally modified by phosphorylation of serine 514 and may represent a mechanism by which its activity can be regulated (Glickman *et al.*, 1997). The Dnmt genes are also subject to transcriptional regulation by methylation. One such example of this is Dnmt1o which has a tissue specific DMR which acts to control transcription of this isoform (Ko *et al.*, 2005). The regulatory region of the tissue specific DMR undergoes developmentally controlled de novo methylation and demethylation while there appears to be no such modification of the Dnmt1s and Dnmt1p 5' region (Ko *et al.*, 2005). However, Doherty *et al.* (2000) found that alterations in Dnmt1 activity in the embryo is not the cause of the aberrant expression of imprinted genes observed after culture. This suggests that although the culture system can influence the methylation status of genes this is not mediated through alterations to the Dnmt enzymes.

3.5.1 Further Work

Although this study did not demonstrate any differences in ovarian *Dnmt* or *Mbd* expression it would be of interest to investigate whether the altered gonadotrophin stimulation results in changes to oocyte gene expression. The analysis of ovarian gene expression described above utilised traditional end-point RT-PCR. This technique only involves the assessment of the product once all the PCR cycles have been carried out. As shown in Figure 3.1 the reaction must be terminated during the exponential amplification phase if meaningful quantitative data is to be obtained. Thus, experiments to establish a suitable number of cycles for each sample must be carried out, which is time consuming and if very low volumes of RNA are available may not be possible. The quantitative analysis of end-point RT-PCR relies on

agarose gel and ethidium bromide which have a poor sensitivity and resolution, such that small changes in expression levels cannot be determined. Real-time RT-PCR has been developed relatively recently and has the benefit of allowing rapid quantification of PCR products. During real-time PCR the reaction products are quantitated after every cycle so the range of exponential amplification can be easily assessed. During the time period when the experiments described in this chapter were carried out the only available PCR technique was the end-point RT-PCR. However, the department has recently acquired a real-time PCR machine utilising the SYBR-green dye assay. The SYBR-green binds to double stranded DNA and once bound the resulting bright fluorescence can be detected and quantified. Use of this real-time RT-PCR system would enable the efficient analysis of *Dnmt* and *Mbd* expression in isolated oocytes from which only small levels of RNA could be extracted and changes in gene expression level may be small. In addition to the *in vivo* approach described in this chapter, an *in vitro* experiment analysing oocyte expression of each *Dnmt* and *Mbd* after exposure to either raised gonadotrophins or steroids could be used to further investigate the possible effect of the hormonal environment on genomic imprinting and DNA methylation.

Chapter 4

Effect of androgens and oestrogens on oocyte global methylation levels

4.1 INTRODUCTION

Hormonally induced changes to DNA methylation have been found in terminally differentiated cell types. One such example, which is of particular interest in relation to the current study, involves alteration to DNA methylation after oestradiol administration, where exogenous oestradiol caused the avian hepatic vitellogenin gene promoter to become hypomethylated (Wilks *et al.*, 1982; Saluz *et al.*, 1986). As previously discussed, in Section 3.5, oestradiol reduces the binding efficiency of avian MDBP-2 to methylated DNA (Bruhat & Jost, 1995; 1996). Subsequent to this, there is demethylation of the vitellogenin gene promoter, starting four hours after oestradiol exposure (Jost *et al.*, 1991). However, there are no previous studies which have investigated the effects of steroids on the DNA methylation levels of the oocyte.

Shi & Haaf (2002) found there was a correlation between the use of superovulation and abnormal methylation patterns in the 2-cell mouse embryo. The percentage of embryos with abnormal methylation levels correlated to the observed embryonic losses during the pre-implantation stages of development. This finding suggests that evaluating the DNA methylation level of an embryo could be a method by which developmental potential could be predicted. Although, embryos are no longer viable after DNA methylation levels have been assessed this technique could be used during the optimisation of culture conditions to determine the impact of different factors on developmental competence. As changes in embryonic DNA methylation levels have been implicated in having a significant impact on development, then carrying out such an analysis on oocytes may prove to be a valid predictor of oocyte

developmental competence. As oocyte quality cannot be determined by visual assessment, the effects of altered environment on the oocyte are difficult to assess. Utilising the potential correlation between DNA methylation level and developmental competence could aid the optimisation of *in vitro* culture conditions. Genomic imprints of the oocyte can be affected when exposed to a period of time in culture, with several imprinted genes demonstrating altered DNA methylation levels (Kerjean *et al.*, 2003). It is not just the imprinted genes which acquire their DNA methylation during oocyte growth. Retro-transposons and regions of satellite DNA must also become correctly methylated, with IAPs (transposable elements) gaining DNA methylation during post-natal oocyte growth (Lucifero *et al.*, 2004). This methylation is thought to be important in ensuring these regions are maintained in a transcriptionally silent state, thus preventing their transposition in the genome (Walsh *et al.*, 1998). In the male germline, these repeat sequences have been shown to undergo re-methylation during embryonic life (Lees-Murdock *et al.*, 2003). However, in the female germline, low methylation levels are maintained during embryonic stages. It is not until the post-natal oocyte growth phase, that the repeat sequences undergo reprogramming of DNA methylation patterns (Lees-Murdock *et al.*, 2003; Kim *et al.*, 2004). Thus, it may be that over the period of oocyte development, when DNA methylation at both maternally imprinted and non-imprinted sequences are laid down, the mechanism is more easily perturbed by inappropriate concentrations of steroids or other factors.

As each imprinted gene and non-imprinted sequence has a particular period of time during oocyte growth over which the methyl groups are laid down, it may be that the

time of exposure to steroids determines which genes are susceptible (Obata & Kono, 2002; Kim *et al.*, 2004; Lucifero *et al.*, 2004). Cultured mouse embryos with aberrant *H19* expression can still have normal *Snrpn* expression (Doherty *et al.*, 2000), suggesting that some imprinted genes may be more sensitive to environmental conditions and demonstrating that aberrant DNA methylation can occur at specific locations rather than be genome wide. In both mice and humans, it appears that much of the DNA methylation is laid down over the later stages of oocyte growth (Lucifero *et al.*, 2004; Geuns *et al.*, 2003). This period of epigenetic reprogramming is comparable to the pre-antral to Graafian stages supported by the culture system routinely used in our laboratory in which late pre-antral intact follicles were cultured in the presence of either raised androgens, raised oestrogens or both steroids raised in combination (Spears *et al.*, 1994; Murray *et al.*, 1998; Spears *et al.*, 1998; Murray *et al.*, 2005). At the end of the culture period oocytes were recovered and used in an IVF procedure. Exposure to raised androgens alone over the period of follicle culture resulted in a higher percentage of recovered oocytes undergoing fertilisation. In contrast, exposure to raised oestrogens, whether alone or in combination with androgens, resulted in fertilisation rates that were lower than controls. Thus, there is a correlation between the time at which oocytes normally undergo DNA methylation reprogramming and the altered steroidal exposure of oocytes *in vitro*, which has previously been found to alter fertilisation rate (Murray *et al.*, 2005). This makes DNA methylation of the oocyte after culture and exposure to steroids a valid mechanism for further investigation.

4.2 AIM

Both the acquisition of maternal genomic imprints and methylation of other non-imprinted and repeat sequences play significant roles in oocyte developmental competence. Thus, this study aims to investigate whether exposure to raised androgens and oestrogens, both *in vivo* and *in vitro*, alters the level of DNA methylation.

Experiment 1:- How does the global DNA methylation level of the oocyte alter during pre-antral to Graafian stage follicular growth?

Experiment 2:- Do raised levels of steroids *in vitro* affect oocyte global DNA methylation?

Experiment 3:- How do global DNA methylation levels of naturally ovulated oocytes compare to those obtained by superovulation?

4.3 MATERIALS AND METHODS

4.3.1 General Chapter Materials and Methods

4.3.1.1 Methylation Immunohistochemistry

Oocytes were fixed in freshly made 4% PFA (Sigma Aldrich, UK) overnight and washed in PBS (Sigma-Aldrich, UK) before being permeabilised in Triton-X100 (0.5% in PBS, Sigma-Aldrich, UK) for 30 minutes at room temperature. This was followed by DNA denaturation (4N HCl, BDH, UK, for 1 hour at 37°C) and a wash with Tween-20 in PBS (0.05%, Sigma-Aldrich, UK) for 30 minutes at room temperature before saturation of non-specific binding sites with BSA (2% in PBS, Sigma-Aldrich, UK) for 1 hour at room temperature. 5-methyl cytosine primary antibody, stored undiluted at 4°C, (Eurogentec, Belgium) was diluted 1:50 in BSA (2% in PBS); 15µl wash and incubation drops were placed in plastic embryo dishes (BD Falcon, USA) and covered with silicon oil (Merck, Germany). Oocytes were moved through the wash drop and into the incubation drop where they were left for 1 hour at 37°C. A wash step was carried out at room temperature for 30 minutes in Tween-20 (0.05% in PBS) prior to the secondary antibody incubation. The secondary was diluted 1:200 in BSA (2% in PBS) and 15µl wash and incubation drops were placed under oil. Oocytes were moved through the wash drop into the incubation drop where they were left for 1 hour at room temperature in the dark. A final wash in PBS for 30 minutes was carried out before oocytes were mounted on superfrost positively charged slides (VWR International Ltd, UK) in vectashield mounting medium (Vector Laboratories, USA). Slides were then stored at 4°C in the dark. Triton X-100, 4N HCl, Tween-20, PBS and BSA solutions were all stored at 4°C for up to one month. Antibodies were not stored at the working dilution, with

the appropriate antibody solutions only being prepared immediately before use. Once the immunohistochemistry protocol was complete slides were stored at 4°C in the dark and images obtained within 6 hours to prevent loss of signal.

4.3.1.2 Propidium Iodide Staining

Fixed oocytes were permeabilised, denatured and washed as for the methylation immunohistochemistry described above up until the saturation of non-specific binding sites. Instead these oocytes were placed in RNase A (2mg/ml PBS, Sigma-Aldrich, UK)) for 1 hour at 37°C before being transferred to PI (25mg/ml, Sigma-Aldrich, UK) for 30 minutes. After a final wash in Tween-20 in PBS (0.05%, Sigma-Aldrich, UK) oocytes were mounted on positively charged Superfrost slides (VWR International Ltd, UK) in vectashield (Vector Laboratories, USA). Samples were observed using the Leica upright TCS-NT confocal microscope with Argon/Krypton laser (Leica Microsystems Ltd, UK) as described in Section 2.9. A small number of oocytes underwent the methylation immunohistochemistry before being counterstained with PI.

4.3.1.3 Methylation Image Analysis and Normalisation of Data

The threshold for the intensity of staining for each experimental run was calculated using the Imaris software package (Bitplane AG, Switzerland). Determination of this threshold value was carried out in the following way. The central image of the Z-stack was identified and the stained regions highlighted using the co-localisation function of the Imaris software which gives a threshold value for the image. The mean threshold for that experimental run could then be calculated. Once the

threshold value had been established all the stored images could be analysed using the Scion Image Software Package (Scion Corporation, USA) as described in Figure 4.1. Comparisons of raw data could only be made within each experimental run, since the intensity of the confocal microscope laser will reduce with age. This means that the level of fluorochrome excitation and therefore level of staining intensity will vary between experimental runs. To allow all data obtained to be compared data was normalised to the mean of the control group. Each oocyte from one culture was compared to the mean of that cultures control group:-

$$1/\text{Mean} \times \text{oocyte total} = \text{normalised value}$$

Once normalised data had been calculated for every experiment these values were plotted on a scattergram.

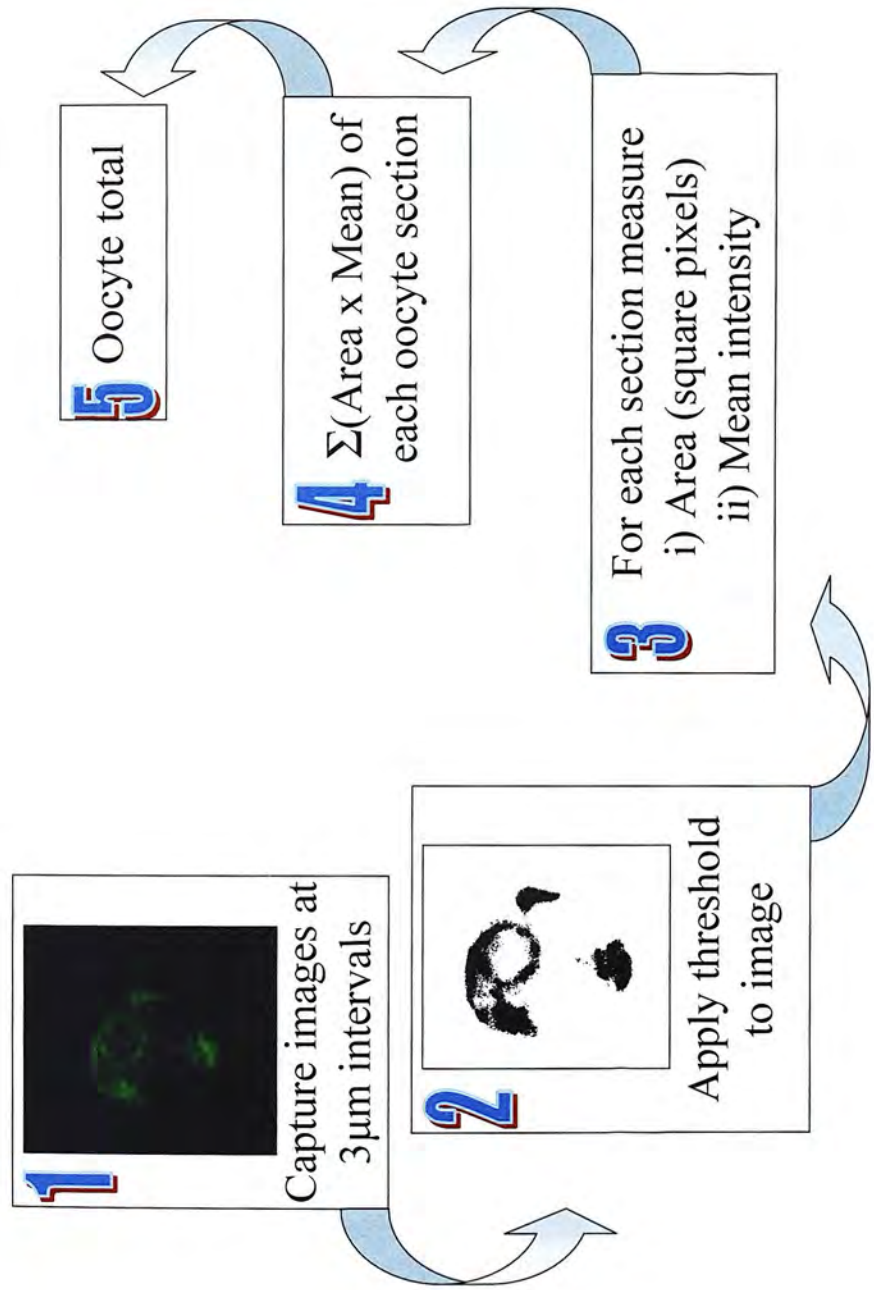


Figure 4.1:- Flow chart illustrating the steps taken to analyse global DNA methylation levels for each oocyte.

4.3.2 Experiment 1:- How does the global DNA methylation level of the oocyte alter during pre-antral to Graafian stage follicular growth?

4.3.2.1 Oocytes from *in vivo* follicles

Four 6 week old F1 females, which had not previously been housed together, were placed with a 12 week old F1 vasectomised male overnight prior to the removal of the ovaries as described in Section 2.4. Females which are housed together tend to have synchronised cycles, while the presence of the male should act as a stimulus to enhance follicular development in any female near to oestrous. Any female which had oocytes in the oviduct, and had therefore already ovulated, was removed from the study. Healthy follicles of sizes comparable to those obtained on Days 0, 2, 4 and 6 of culture were dissected from the remaining ovaries (Section 2.5). Thus, follicles of the following sizes were obtained 175 ± 2μm (Day 0); 200 ± 2μm (Day 2); 290 ± 2μm (Day 4) and 340 ± 2μm (Day 6). These follicles were ruptured and the recovered oocytes fixed overnight in 4% PFA, as detailed in Section 2.6.2, prior to methylation immunostaining (as described in Section 4.3.1.1).

4.3.2.2 Oocytes from cultured follicles

Pre-antral follicles were dissected from 3 week old F1 mouse ovaries and cultured for 0, 2, 4 or 6 days in standard medium (described in Sections 2.4 and 2.5). The basic culture medium and system used has previously been described in Section 2.7.1 and 2.7.2. Follicles removed from culture were ruptured and the oocytes denuded of cumulus cells by repeated pipetting (Section 2.6.1). Oocytes were immediately fixed in fresh 4% PFA overnight, before being stored in PBS until the end of the 6 day culture period. Subsequently, all oocytes from the culture were stained with either 5-

methylcytosine antibody or propidium iodide (PI) as detailed in Sections 4.3.1.1 and 4.3.1.2. A small number of oocytes were counterstained with PI after the methylation immunostaining. Only a visual assessment of the methylation levels was carried out for these oocytes.

4.3.3 Experiment 2:- Do raised levels of steroids *in vitro* affect oocyte global methylation?

As described previously in Section 2.5, pre-antral follicles were dissected from 3 week old F1 ovaries and cultured for 5 days. Cultures were carried out as previously described in Section 2.7, but the standard medium detailed in Section 2.7.1 was supplemented after filtration with Arimidex and/or DES which had been solubilised in ethanol (ETOH). The treatment groups are detailed in Table 4.1 below and are identical to those determined by previous work in the laboratory and detailed in Murray *et al.* (2005). DES acts to raise the oestrogen levels present in the media, however this treatment had no effect on the androgen levels. DES is a non-steroidal oestrogen with a higher affinity to both ERα and ERβ than oestradiol (Kuiper *et al.*, 1997; Bolger *et al.*, 1998; Nikov *et al.*, 2001). The binding of DES to the ERs results in conformational changes to the receptor that are similar to those observed when oestradiol is the ligand (Nikov *et al.*, 2001). In addition, the ER-DES complex has a comparable binding affinity for the oestrogen response elements as the ER-oestradiol complex in both humans and *Xenopus* (Nikov *et al.*, 2001). When *in vitro* matured mouse COCs are exposed to DES the oocytes have a delayed cell cycle progression while at high doses of DES an abnormal condensed chromosome structure is observed. However, there is no apparent alteration to germinal vesicle stage oocytes

or GVBD even at the highest doses of DES used (Can & Semiz, 2000). The androgen levels were altered by the Arimidex, a non-steroidal aromatase inhibitor which has been shown to have no estrogenic or anti-estrogenic effects (Lønning *et al.*, 1998; Section 1.15). This raises androgens by preventing their conversion to oestrogens in the GCs of the follicle. This treatment has the double effect of raising androgens and also lowering the level of oestrogens (as shown in Figure 1.10 A and B). At the end of culture period follicles were ruptured and the oocytes were fixed at 4°C overnight in fresh 4% PFA for use in the methylation immunohistochemistry (Sections 2.6.1 and Section 4.3.1.1). Methylation levels were obtained using the confocal microscope (Section 2.9) analysed and normalised to allow comparisons between experimental runs. Chromatin structure was assessed for each treatment groups and the number of SN and NSN were recorded. Oocytes exposed to A+E were obtained by carrying out five repeats of the culture system, while three replicates of the A only and E only cultures were carried out.

Table 4.1:- The raised steroid treatment groups used in culture

Treatment Group	Arimidex	DES
Raised androgen (A only)	0.1μm	~
Raised oestrogen (E only)	~	4nmol
Raised androgen & oestrogen (A+E)	0.1μm	4nmol

4.3.3.1 Permeabilisation Test

To confirm that any observed alterations in global DNA methylation levels were a true change and not a result of the treatment affecting the permeability of the oocyte, the ability of the secondary antibody to permeate the oocyte after culture was tested. Oocytes from the control and A+E treatment groups were used in the normal methylation immunohistochemistry protocol, with the exception of the primary antibody incubation. After exposure to the secondary antibody, the oocytes were only briefly washed in Tween-20 in PBS (0.05%, Sigma-Aldrich, UK) before being mounted on slides. The level of secondary antibody within the oocyte was assessed using the Leica confocal microscope and software (Section 2.9).

4.3.4 Experiment 3:- How do global DNA methylation levels of naturally ovulated oocytes compare to those obtained by superovulation?

To obtain naturally ovulated oocytes, 6 six week old F1 females were caged overnight with 12 week old F1 surgically vasectomised males. The following morning the ovaries and oviducts were removed post-mortem into sterile dissecting medium (as described in Sections 2.2 and 2.4). The oviducts were ruptured using insulin syringes and the COCs retrieved (Section 2.6.2). These were transferred into fresh dissecting medium supplemented with hyaluronidase (300μgml^{-1}, Sigma-Aldrich, UK) to remove the cumulus cells from the oocytes. Superovulated oocytes were obtained from 3 six week old F1 females, as described in Section 2.3. All oocytes were fixed overnight at 4°C in fresh 4% PFA prior to methylation immunostaining as described above (Section 4.3.1.1). As described in Section 4.3.1.3, methylation levels were analysed and the data normalised to the mean of the

naturally ovulated group. Three repeats of this experimental protocol were carried out and the results analysed separately.

4.3.5 Statistics

Results from Experiment 2, analysis of DNA methylation levels after exposure to raised steroid levels in culture, were analysed using nonparametric ANOVA and were subsequently analysed using Dunn's multiple comparison test. The results of the chromatin configuration analysis were analysed using Chi-Square tests.

The results within each experimental run of Experiment 3, comparing DNA methylation levels of naturally ovulated and superovulated oocytes, were compared by unpaired t-tests with Welch correction.

4.4.1 Experiment 1:- How does the global DNA methylation level of the oocyte alter during pre-antral to Graafian stage follicular growth?

4.4.1.1 Oocytes from *in vivo* follicles

Oocytes from *in vivo* derived follicles, of comparable size to those previously obtained from culture, showed an increase in global DNA methylation level over the period of oocyte growth and development (Figure 4.2). These oocytes have a diffuse pattern of staining at the pre-antral stage, but the staining around the nucleolus in oocytes from larger *in vivo* follicles is less defined than that seen after culture.

4.4.1.2 Oocytes from cultured follicles

Analysis of the images from the initial control cultures showed that over the culture period the global methylation levels of the oocytes increased considerably (Figure 4.3, Swales & Spears, 2005). In addition to the change in global DNA methylation level, the pattern of staining can also be seen to alter with oocyte development. On Day 0 the staining is diffuse, while by Day 6 the pattern is more compact and there is a well defined region of strong staining around the nucleolus. The chromatin levels and pattern also changed over the culture period in a manner comparable to that of the methylation staining (Figure 4.4). In oocytes which were counterstained with PI, after immunostaining for DNA methylation, the two stains were found to have an almost identical localisation pattern (Figure 4.5).

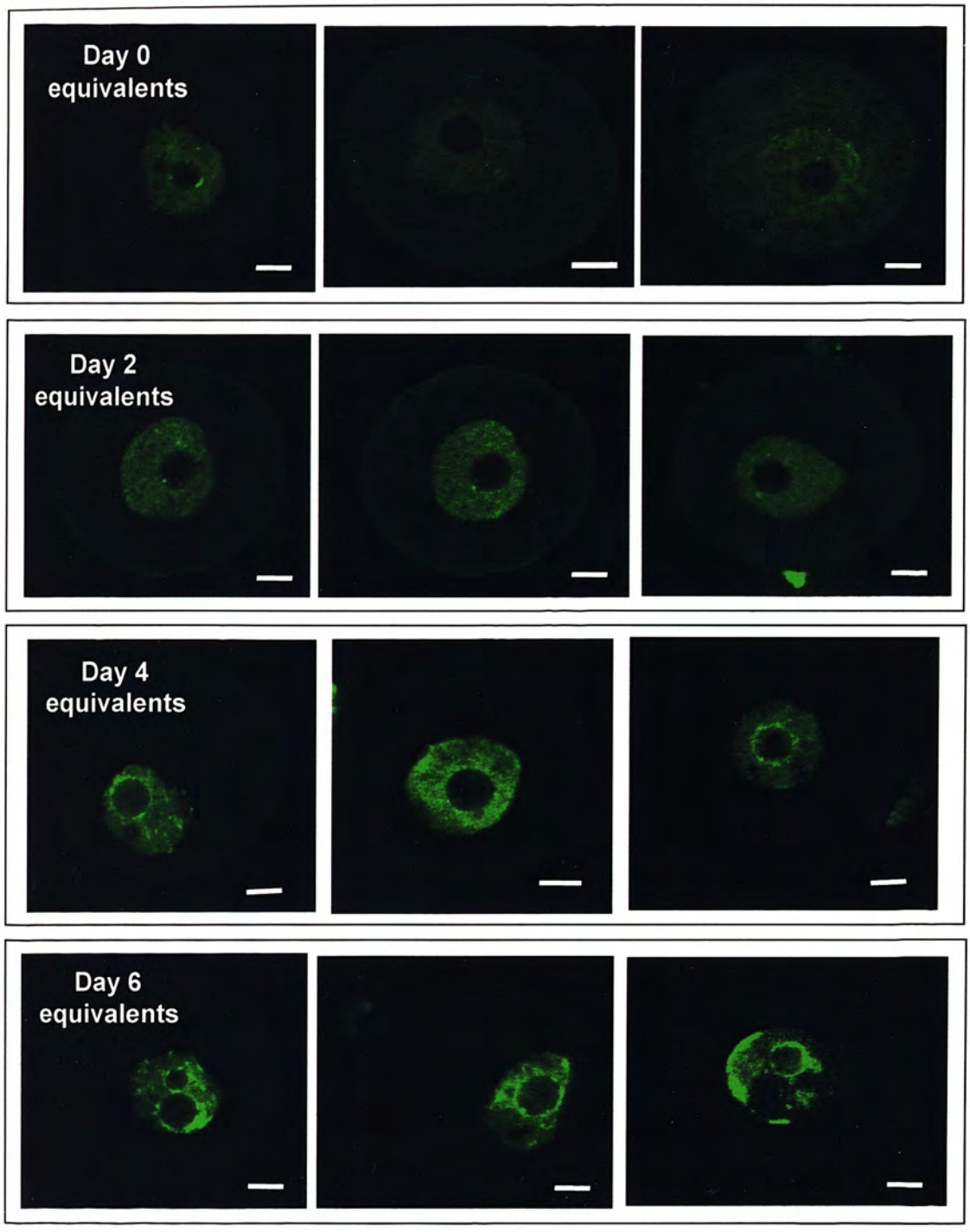


Figure 4.2 – Global DNA methylation level of oocytes increases over the period of growth and development associated with the pre-antral to Graafian stages *in vivo*. Oocytes are from follicles of equivalent size to Day 0, 2, 4 and 6 in culture. Follicles were dissected from the ovaries of four 6 week old F1 mice. Three replicates of this experiment were carried out. Day 0 equivalent (175 ± 2μm) n=18; Day 2 equivalent (200 ± 2μm) n=14; Day 4 equivalent (290 ± 2μm) n=9; Day 6 equivalent (340 ± 2μm) n=14. The scale bars represent 10μm.

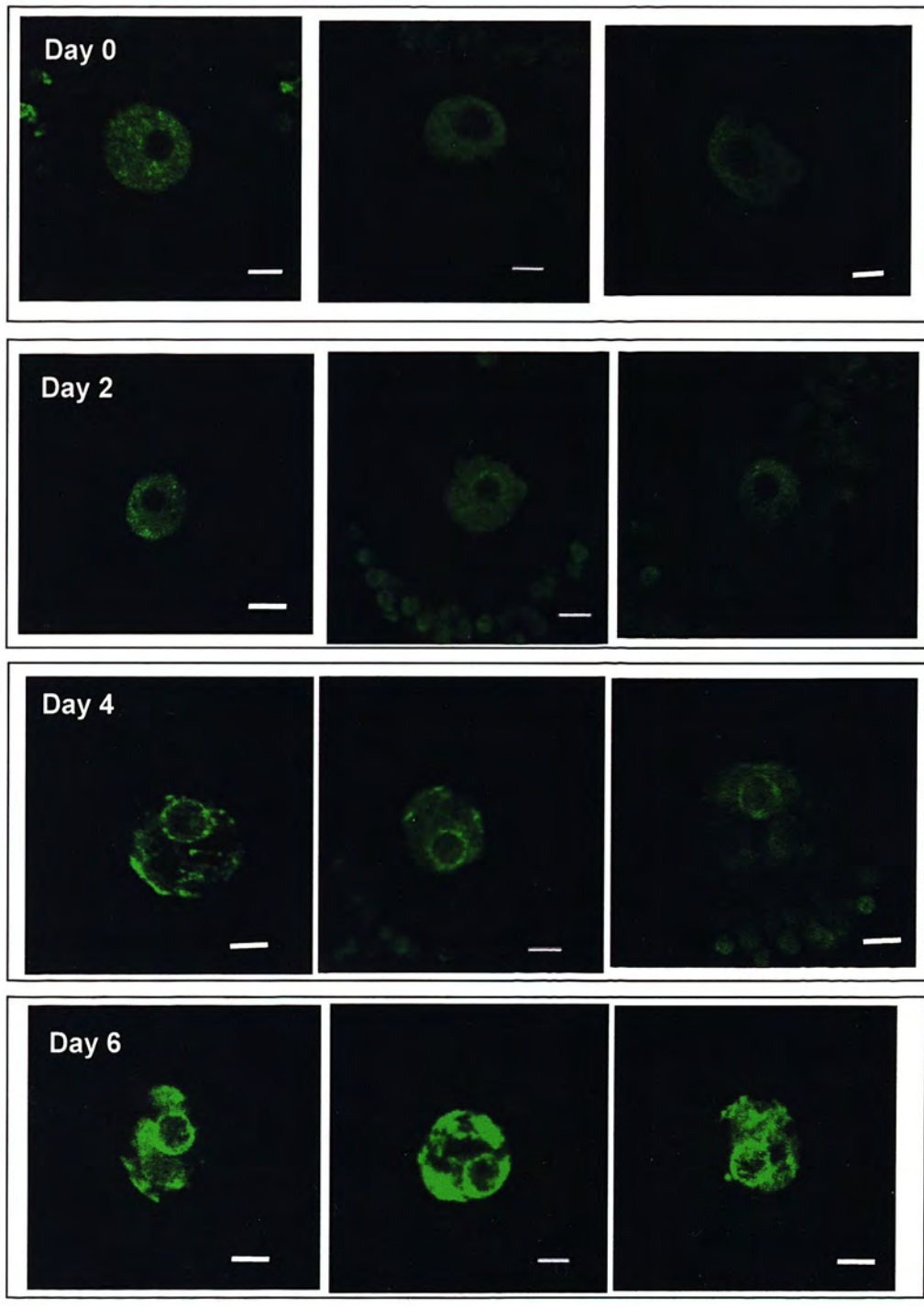


Figure 4.3:- The global DNA methylation level of oocytes increases over the period of growth and development associated with follicle development from pre-antral to Graafian stages in culture. Follicles were dissected from the ovaries of four 3 week old F1 mice and randomly assigned to each time point. Two repeats of this culture system were carried out. Confocal images of global DNA methylation levels of oocytes removed from culture on Day 0 (n=8), Day 2 (n=8), Day 4 (n=8) and Day 6 (n=10) and stained using a 5-methyl-cytosine antibody and FIT-C secondary. The scale bars represent 10μm.

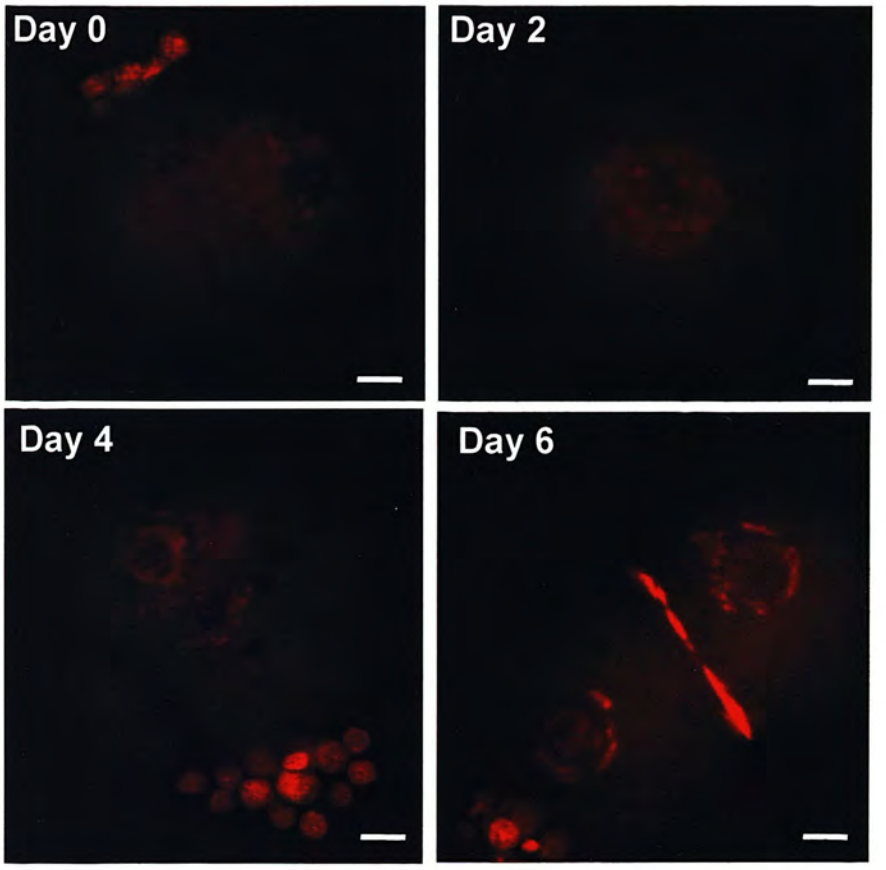


Figure 4.4 – Confocal images showing chromatin of oocytes over the period of growth and development associated with follicle development from pre-antral to Graafian stages in culture. Follicles were dissected from the ovaries of four 3 week old F1 mice and randomly assigned to each time point. Two repeats of this culture system were carried out. Oocytes were removed from culture stained with propidium iodide on Day 0 (n=8), Day 2 (n=10), Day 4 (n=10) and Day 6 (n=11). The scale bars represent 10μm.

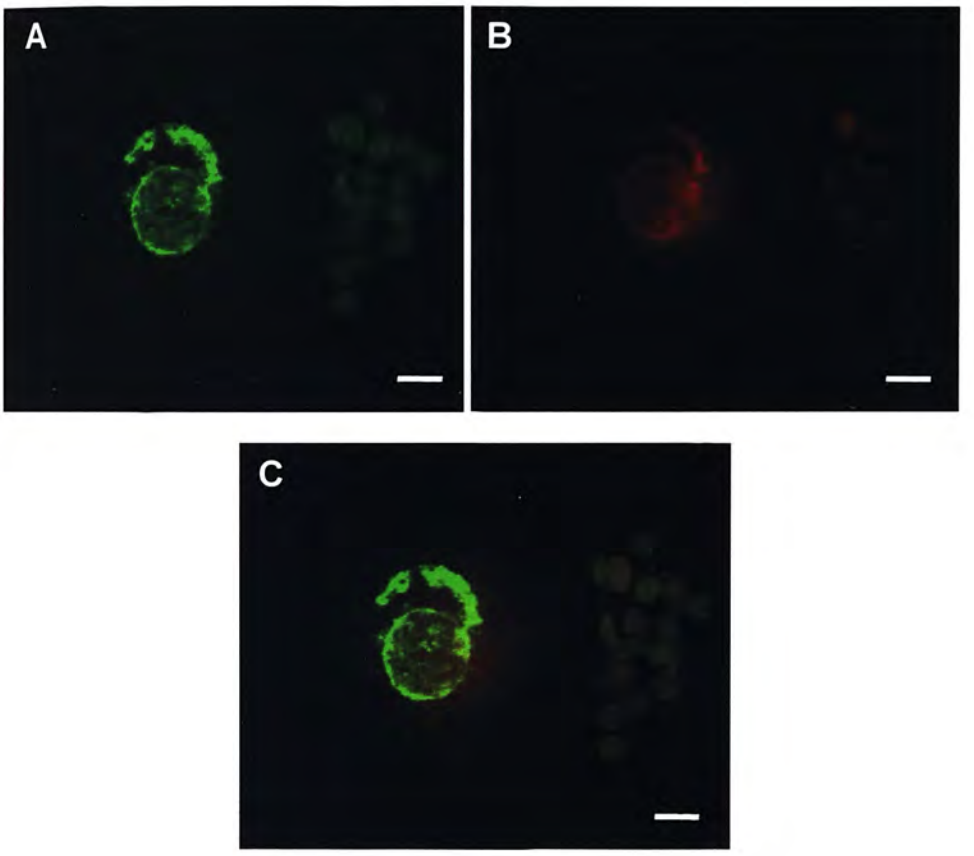


Figure 4.5:- Confocal images of an oocyte from a Day 6 cultured follicle immunostained for methylation before counterstaining with propidium iodide (PI). The almost identical localisation of both the methylation and chromatin stains can be seen. **A)** Image showing methylation channel only: **B)** Image showing PI channel only: **C)** Image showing an overlay of both channels. Follicles were dissected from the ovaries of four 6 week old F1 mice and cultured. Two repeats of this culture were carried out. At the end of culture the oocytes were divided between several immunohistochemistry protocols with 6 oocytes allocated to this methylation and PI staining group. The scale bars represent 10μm.

4.4.2 Experiment 3:- Do raised levels of steroids *in vitro* affect oocyte global methylation?

Differences in global DNA methylation level were observed after exposure to raised steroids (Figures 4.6 and 4.7). There was a significant increase ($P< 0.0001$) in methylation after A+E treatment while in the A only group there was a significant decrease ($P<0.001$) in global methylation level. The E only treatment group did not give a result which significantly differed from the control group. The effect of each treatment group on global DNA methylation levels was consistent in all runs of this experiment. Thus, the data from all the experimental repeats was combined and analysed together. When chromatin configurations were assessed more than 80% of oocytes in all treatment groups had a SN chromatin arrangement (Figure 4.8). There was a significant difference ($P=0.03$) in the proportions of SN and NSN oocytes between the treatment groups. Further analysis which compared each treatment group to the controls found that the distribution of oocytes in the SN and NSN chromatin configuration patterns only differed significantly from controls after A+E exposure ($P=0.009$).

When compared to controls oocytes from the A only group have a reduced level of DNA methylation and a lower proportion of oocytes with an SN configuration. This observation is true for all the treatment groups used in this study with the changing pattern of DNA methylation levels between treatments being paralleled by the percentage of oocytes with an SN chromatin configuration (Figures 4.7 and 4.8).

4.4.2.1 Permeabilisation test

This assessment was qualitative thus there were no statistics used. The assessment used the image software's ability to assess intensity along a user defined line. A line was drawn through the oocyte passing through the centre of the nucleolus (the section assessed was that in which the oocyte had the greatest diameter). Qualitative assessment was made as to whether the line showed the presence of altered staining across the oocyte (as would be the case if altered penetration of the GV was occurring in response to steroid exposure). The majority of oocytes examined had an unstained nucleolus but there was no evidence of differential staining of the GV (Figure 4.9). There was no difference in the level of secondary antibody in the control and A+E group oocytes. This demonstrated that exposure to the raised steroids did not alter the permeability of the zona pellucida and the ability of the antibody to enter the oocyte. Thus, the difference in staining intensity does represent a true alteration in methylation level, rather than demonstrating an artefact of the treatment.

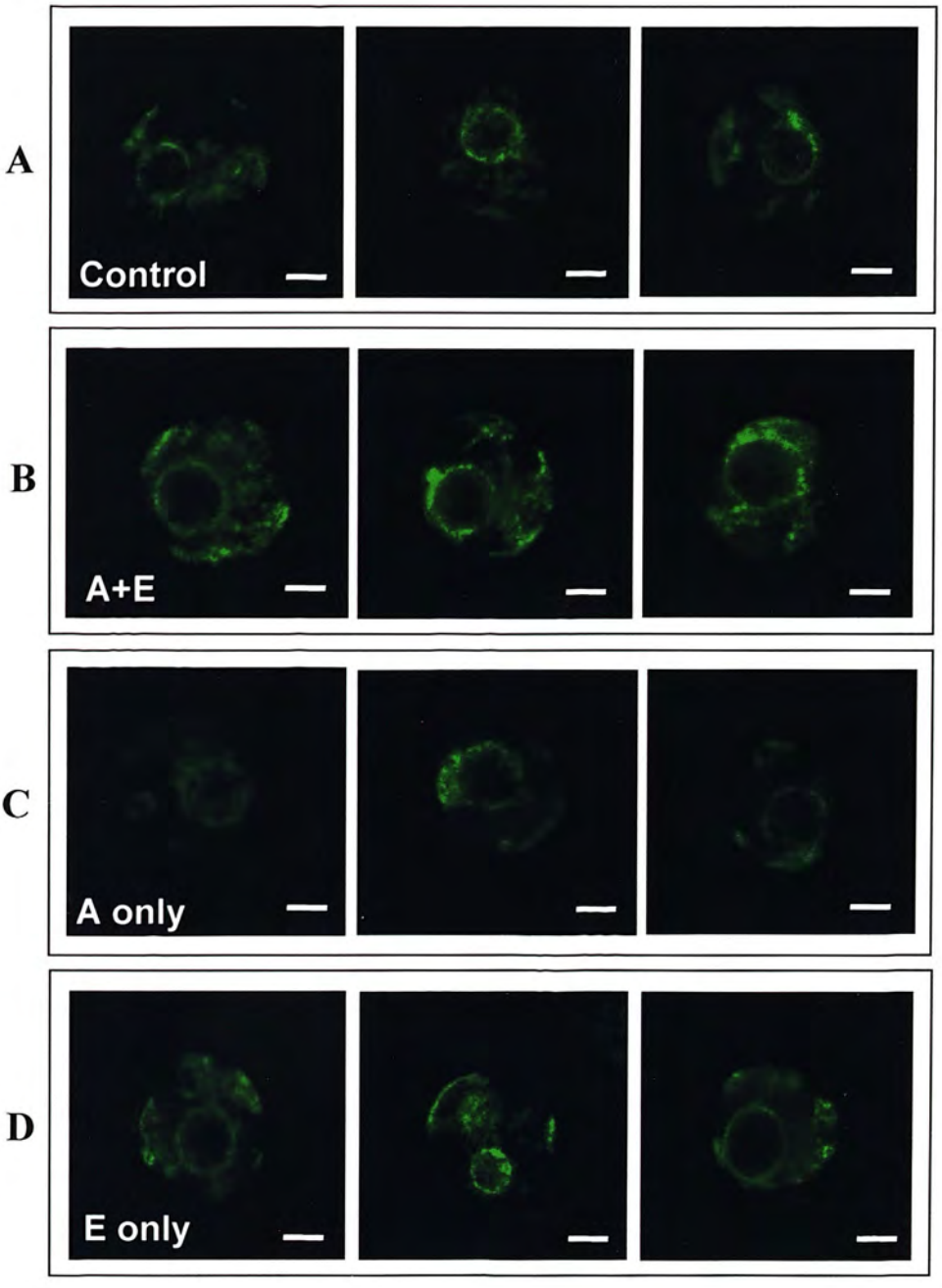


Figure 4.6:- Confocal Images of oocytes stained using the anti-5-methylcytosine antibody after exposure to different steroid treatments in culture. Panel A are oocytes from the control cultures (n=114). Panel B are images of oocytes recovered from follicles cultured with high A+E (n=61). Panel C are oocytes from A only cultures (n=51) and Panel D are oocytes from E only cultures (n=48). Oocytes exposed to A+E were obtained by carrying out 5 repeats of the culture, while three replicates of the A only and E only cultures were carried out. Each run of the culture system consisted of ovarian follicles dissected from a minimum of three 3 week old F1 females randomly distributed between the treatment groups. A=androgens; E=oestrogens. The scale bars represent 10μm.

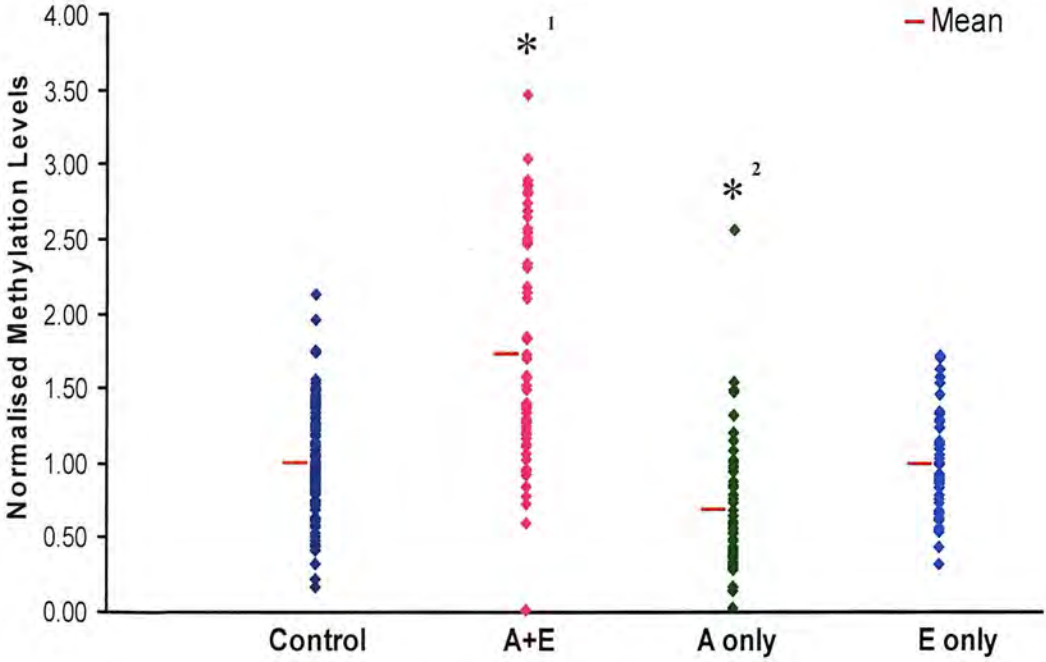


Figure 4.7:- Normalised global DNA methylation levels for each oocyte according to treatment group, Control, High Androgen (A only), High oestrogen (E only) and high androgen and oestrogen in combination (A+E). Horizontal red bars represent the mean of the treatment group. Control n=114; A+E n=61; A only n=51; E only n=48. Oocytes exposed to A+E were obtained by carrying out 5 repeats of the culture, while three replicates of the A only and E only cultures were carried out. Each run of the culture system consisted of ovarian follicles dissected from a minimum of three 3 week old F1 females randomly distributed between the treatment groups.
$*^{1}$ represents a significant difference ($P<0.0001$)
$*^{2}$ represents a significant difference ($P<0.001$)

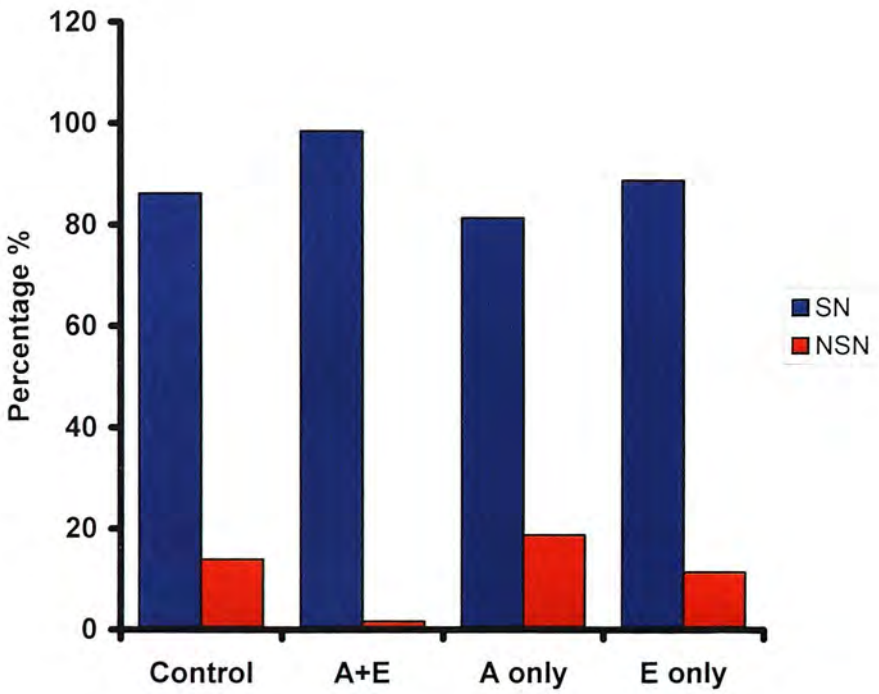
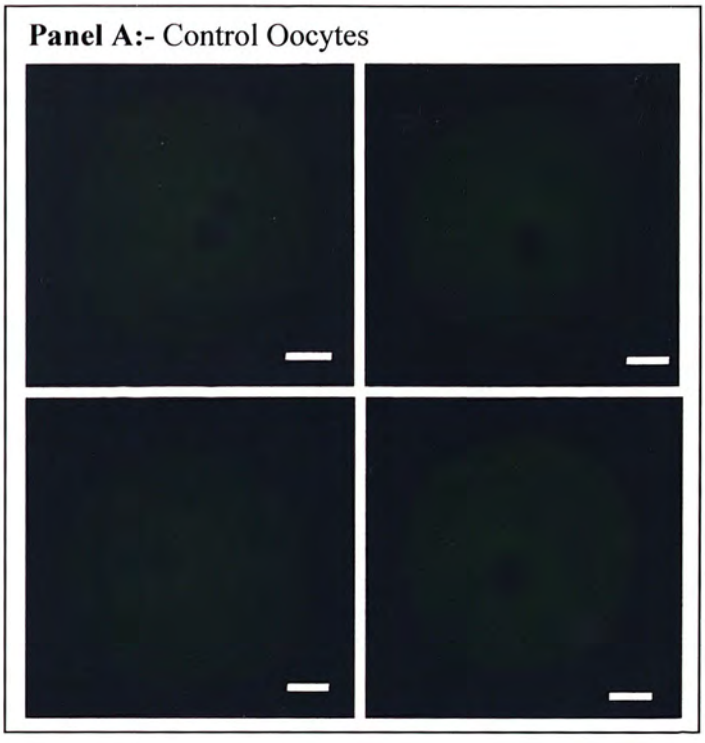


Figure 4.8:- Analysis of chromatin configuration by treatment group, Control, high androgen (A only), high oestrogen (E only) and high androgen and oestrogen in combination (A+E). When compared to the controls only the A+E treated oocytes had a significantly different distribution of SN and NSN oocytes (P=0.009). Oocytes exposed to A+E were obtained by carrying out 5 repeats of the culture, while three replicates of the A only and E only cultures were carried out. Control n=114; A+E n=61; A only n=51; E only n=48. Each run of the culture system consisted of ovarian follicles dissected from a minimum of three 3 week old F1 females randomly distributed between the treatment groups. SN – Surrounded Nucleolus; NSN – Non-surrounded Nucleolus.



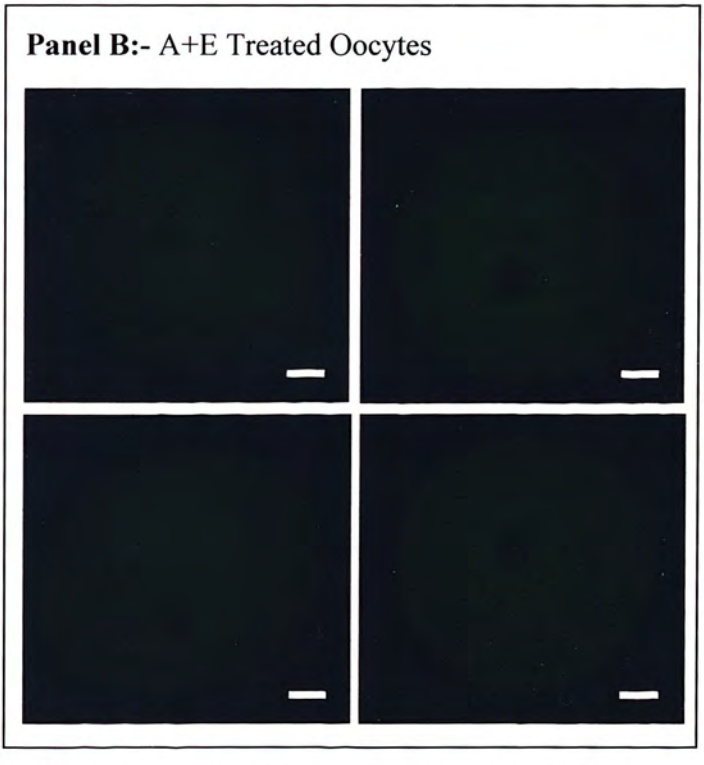


Figure 4.9:- Images of oocytes from permeability test experiment with Panel A showing the control group and Panel B the high androgen + oestrogen group. Follicles were dissected from the ovaries of four 3 week old F1 mice and randomly assigned to either the control (n=25) or A+E (n=18) treatment group. Two repeats of the culture system were carried out. Confocal images of oocytes removed from culture on Day 5 and stained with FIT-C secondary were obtained to assess any alteration in antibody accessibility to the germinal vesicle. The scale bars represent 10μm.

4.4.3 Experiment 3:- How do global DNA methylation levels of naturally ovulated oocytes compare to those obtained by superovulation?

As can be seen in Figure 4.10**A**, in the first run of this experiment the oocytes obtained after superovulation had slightly lowered global DNA methylation levels. This reduction in DNA methylation level was not found to be significant. Moreover, when the experiment was repeated (Figure 4.10**B**) the superovulated oocytes had significantly higher ($P<0.0001$) global DNA methylation levels than oocytes recovered after natural ovulation. In contrast the superovulated oocytes from the third run of this experiment had a lower staining intensity than those obtained after natural ovulation. In fact, this difference was so great that images suitable for comparison could not be obtained. To allow relative methylation levels to be assessed all images must be obtained with the same confocal microscope settings, including a consistent detection level. It was not possible to obtain images from both the natural and superovulated treatment groups utilising one detection level, as this led to saturated staining of the naturally ovulated oocytes but no readable staining of the superovulated group. Thus, no relative comparison between the two treatment groups could be carried out. As the results from this experiment did not demonstrate a consistent pattern as to whether superovulation increased or lowered global DNA methylation levels the data from each run was analysed separately.

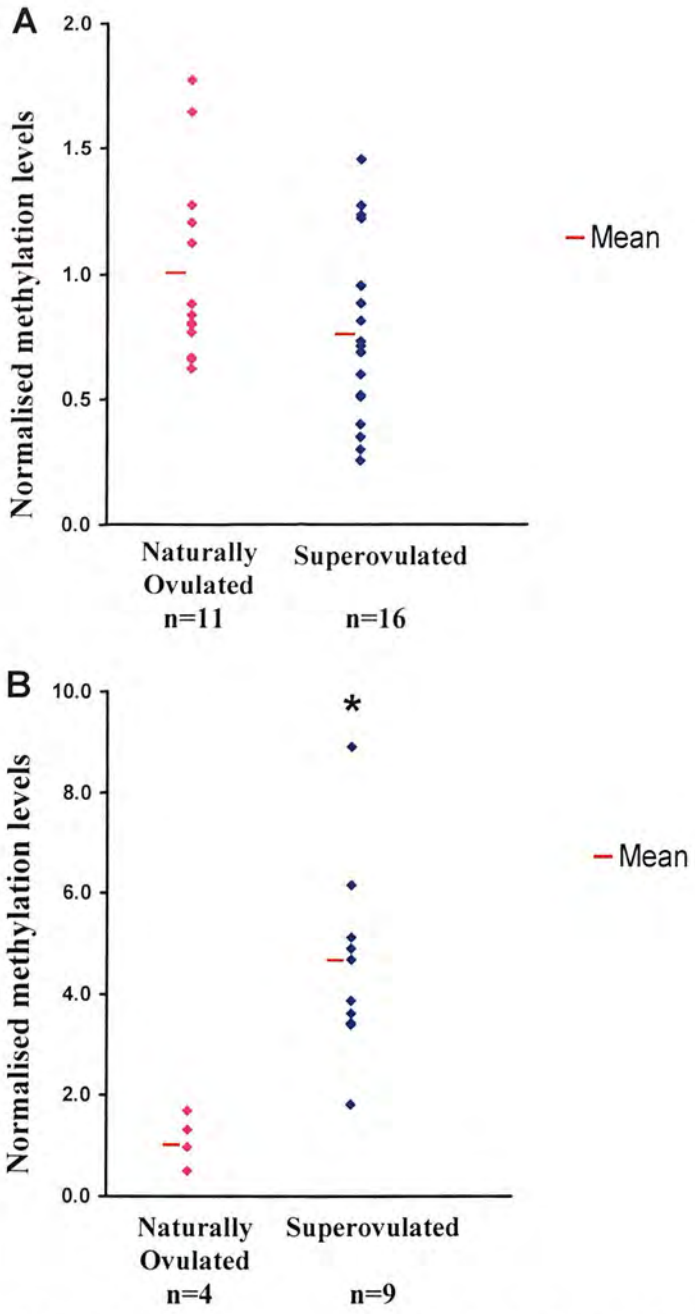


Figure 4.10:- Normalised global DNA methylation levels for each oocyte according to whether it underwent superovulation or natural ovulation. Superovulated group – for each experimental run, oocytes from three 6 week old F1 females were recovered, pooled and a random sample used in immunohistochemistry. Naturally ovulated group – oocytes were recovered from 6 week old F1 females: **A)** first experimental run: oocytes obtained from 2 females; **B)** second experimental run: oocytes obtained from 1 female. Data is not shown for the third experimental run as the intensity differences between the two groups were too great for images to be obtained using identical confocal parameters. Horizontal red bars represent the mean of the group. * represents a significant difference ($P<0.0001$).

4.5 DISCUSSION

Over pre-antral to Graafian follicle development, oocytes from cultured follicles and those derived from unstimulated *in vivo* follicles, exhibit increasing levels of global DNA methylation. This finding is in accordance with the laying down of maternal imprints and the methylation of non-imprinted repeat sequences during oocyte development reported by other groups (Obata & Kono, 2002; Lucifero *et al.*, 2004). The method used in the current study does not differentiate between the DNA methylation at imprinted gene loci and that present on other non-imprinted sequences. Imprinted genes account for a relatively small proportion of the total methyl groups present on the genome, with the vast majority of methyl groups being present on methylated repeat sequences. As the observed increase in global methylation level over oocyte development, both in culture and *in vivo*, is large, it is unlikely to be purely due to the methylation of imprinted genes. The sensitivity of the anti-5-methylcytosine antibody and the regions it binds to must also be taken into account when assessing the results of these experiments. The immunochemical assessment of global DNA methylation patterns has been assisted by the development of anti-5-methylcytosine antibodies. Originally rabbit polyclonal antibodies were developed against 5-methylcytosine conjugated to BSA and these antibodies suggested that satellite DNA on the juxtacentromeric long arms of chromosomes 1, 9, and 16 is heavily methylated in humans, while the rDNA repeats on the acrocentric short arms of group D chromosomes (13, 14, and 15) and group G chromosomes (21 and 22) are largely unmethylated. Mouse satellite DNA is also heavily methylated and easily detectable with anti-5-methylcytosine antibodies (Miller *et al.*, 1974). However, these polyclonal antibodies are difficult to raise and

have to be constantly re-derived and assessed. This hurdle was reduced with the development of a mouse monoclonal to 5-methylcytosine (anti-5mC) by Niveleau *et al.* (1992), which had less problems with non-specific binding to DNA and while still a semi-qualitative approach, gave reproducible assessments of the patterns and amounts of DNA methylation in cell lines and oocytes and embryos. As chromosomal regions with a high density of methylated cytosines will bind more of the anti-5mC the strength of the signal can be used to assess the distribution and level of DNA methylation at CpG dinucleotides (Dahl & Guldberg, 2003). A large proportion of the methylated cytosines are located in the different repeat sequences of the genome. Thus, staining with anti-5mC largely reflects the methylation present in interspersed repetitive sequences (Mayer *et al.*, 2000). A high level of binding of anti-5mC has been demonstrated on human metaphase chromosomes where the antibody binds to the repetitive DNA sequences (De Capoa *et al.*, 1998). Further analysis of the staining patterns of chromosomes was carried out using both anti-5mC and a probe for the satellite regions of DNA. On metaphase chromosomes there was found to be a large region of overlap between the signals from these two markers, whereas there was a smaller degree of overlap on the chromosome spreads of interphase nuclei (De Capoa *et al.*, 1998). This finding was also confirmed in fibroblast cell lines where there was a colocalisation between heterochromatic regions and anti-5mC binding (De Capoa *et al.*, 1999). Within cultured lymphocytes there are four types of binding site that have been identified for anti-5mC (Barbin *et al.*, 1994). These are Type I sites which correlate to the secondary constrictions and a few juxtacentromeric regions. The Type II sites are defined as those corresponding to the T-bands while Type III sites are considered to form an R-band pattern. The

binding of anti-5mc to the short arms of acrocentrics makes up the final Type IV sites (Barbin *et al.*, 1994). The intensity of staining of these sites has been found to differ with both Type I and II sites being strongly stained, while the signal from Type III sites is weaker. Although the Type IV sites have the strongest anti-5mC signal this region demonstrates polymorphic staining (Barbin *et al.*, 1994). The fact that these differing patterns of anti-5mC binding can be observed across the different chromosomal regions represents the uneven distribution and localisation of the methylated cytosines (Barbin *et al.*, 1994). The staining of bovine fibroblast cells with anti-5mC demonstrates that the binding of this antibody is not restricted to the heterochromatic regions. There is also staining of the euchromatin regions present in a banding pattern (Bourc'his *et al.*, 2001).

During oocyte development there are changes to the chromatin configuration of the oocyte, which constitutes an important process in oocyte nuclear maturation (Section 1.6). The pattern of methylation staining seen in this study was identical to the PI staining of chromatin. Thus, in addition to analysis of methylation levels, the chromatin configuration in these images was also assessed. From these results, oocytes from pre-antral follicles demonstrate a more diffuse chromatin pattern, with no defined region of chromatin condensation surrounding the nucleolus. All the oocytes from Day 6 cultured follicles had more condensed chromatin and a SN. These observations support the previously published data describing a diffuse chromatin distribution being characteristic of transcriptionally active immature oocytes, prior to acquisition of meiotic competence (Mattson & Albertini, 1990; Wickramasinghe *et al.*, 1991; Bouniol-Baly *et al.*, 1999). The more condensed SN

configuration correlates with oocyte maturation, cessation of transcription and the ability to undergo germinal vesicle breakdown (Zuccotti *et al.*, 1998; Bouniol-Baly *et al.*, 1999; Liu & Aoki, 2002). Oocytes which have achieved the SN chromatin configuration have increased developmental competence (Zuccotti *et al.*, 1998).

When the *in vivo* oocytes obtained from the Day 6 equivalent follicles were analysed they had a less condensed chromatin structure than those oocytes isolated from comparable cultured follicles. Additionally, not all *in vivo* derived oocytes were found to have a SN. This suggests that there are differences in the acquisition of developmental competence between cultured and *in vivo* oocytes. Although this may represent a precocious maturation of oocytes from cultured follicles, it has been proposed that premature acquisition of SN chromatin configuration may be associated with initiation of atresia (Zuccotti *et al.*, 1998; Bouniol-Baly *et al.*, 1999; Liu & Aoki, 2002). The differences in both DNA methylation level and pattern between oocytes from *in vivo* and *in vitro* follicles of comparable size may represent an alteration in developmental timing of the oocyte rather than an inherent change in the acquisition of DNA methylation.

The culture system described here had previously been used to provide oocytes for IVF, which allowed investigation of the effects of raised steroid levels on both fertilisation rate and embryo development (Figure 1.11 and Murray *et al.*, 2005). With the exception of the E only group, this study found an inverse relationship between global DNA methylation levels and fertilisation rate. The A only group had the lowest DNA methylation level but the highest fertilisation rate, while the reverse

was true for the A+E treatment. The E only group did not support this pattern. In this treatment group, the fertilisation rate is significantly lower than that of the control group, but there is no significant difference between the global methylation levels for these two groups. When considering the results from this experiment it must be noted that the control group does not represent oocytes exposed to the "normal" physiological environment. Thus, any deviation from the control levels of DNA methylation may not indicate a detrimental effect of the steroid treatments. Indeed, when the previous IVF fertilisation rates (Murray *et al.*, 2005) are considered it would appear that those oocytes exposed to androgen during culture may in fact have matured in a physiologically more "normal" manner. The fact that the same culture protocol and treatment parameters were employed in both the current and previous study (Murray *et al.*, 2005) allows valid comparisons to be drawn. The previous IVF results were obtained from three experimental replicates, each demonstrating a consistent pattern, thus illustrating the reproducibility of the experimental procedure and protocol. Additionally, the DNA methylation level and embryo developmental capacity cannot both be measured for an individual oocyte. Therefore these assessments will always have to be made on a separate pool of oocytes. Although the control group has a large spread of values it is still valid to normalise all results to the mean of this group. It was vital to have a common point of reference to allow a comparison between experimental runs. The spread of results in the control group does not detract from the findings in this section as it is a comparative study rather than giving absolute values.

Interestingly, there is a correlation between the DNA methylation levels and the proportion of oocytes with a SN chromatin configuration. After A+E treatment methylation levels are higher than those of control oocytes. There is a concurrent increase in the proportion of oocytes with a SN than seen in the control group. The A only treatment gave both the lowest percentage of SN oocytes and the lowest global methylation levels. This trend may represent subtle differences in the developmental competence of the oocytes within each treatment group, which could not be determined by assessment of follicular health. Chromatin configuration, DNA methylation and gene transcription are three connected processes. As previously discussed in Section 1.12.3, the addition of methyl groups to DNA allows binding of the relevant Mbd protein and co-repressor complexes, which subsequently recruit HDACs. The deacetylation of the histones then results in condensation of the chromatin structure, thereby repressing gene transcription. When the results of this study are considered, there is a correlation between lowered methylation levels and a reduction in the percentage of oocytes with the more condensed SN chromatin configuration. Conversely, hypermethylated DNA is associated with more oocytes achieving condensed chromatin. This finding is supported by previous studies which found that DNA methylation plays an important role in the condensation of chromatin, such that if DNA becomes hypomethylated then heterochromatin is under condensed (Maraschio *et al.*, 1992; Almeida *et al.*, 1993; Haaf & Schmid, 2000). In this study it may the case that steroid-induced changes in DNA methylation level impacts on subsequent chromatin remodelling, thereby altering gene transcription. If in the A+E group the increase in DNA methylation has resulted in precocious chromatin condensation, it may mean that gene transcription was prematurely halted.

In this case, the oocyte will not have the appropriate mRNA stores in place to support fertilisation or pre-implantation development. Alternatively, it may be that both the altered methylation levels and the SN/NSN ratio after steroid treatment could be the outcome of altered timing of oocyte development, rather than specific effects of steroids on DNA methylation or chromatin configuration. Intriguingly, the A only group which had the highest proportion of oocytes at the NSN stage (which is the configuration associated with reduced maturity), was the group with a significantly raised fertilisation rate. This may add weight to the proposal that premature entry into SN is a negative parameter in developmental competence potentially due to impending atresia (Zuccotti *et al.*, 1998; Bouniol-Baly *et al.*, 1999; Liu & Aoki, 2002). Although the results from this experiment demonstrate that the steroidal environment can influence the DNA methylation level of the oocyte it is not possible to tell how this interaction occurs or whether this change influences later embryo development.

The superovulatory gonadotrophins act to recruit follicles into the pre-ovulatory cohort. The development of immature growing follicles is accelerated by the increased levels of FSH. This FSH will also have resulted in the rescue of follicles which would otherwise be destined for atresia. This may result in oocytes being ovulated from follicles in which atretic pathways had already been activated, prior to the addition of exogenous FSH. When this is taken into account, it may be anticipated that there would be differences in the quality of oocyte recovered after superovulation, when compared to natural ovulations. The results presented here do suggest that one important aspect of oocyte maturation, the acquisition and

maintenance of DNA methylation, may differ between the two groups of oocytes, but found tremendous variability in that effect. The altered average DNA methylation levels observed in the superovulated oocytes may not represent a direct effect on DNA methylation levels by the altered hormonal environment. It may be that the follicles stimulated to ovulate contained oocytes which had not yet had the complete maternal imprints laid down or were lacking methylation on some of the normally methylated repeat sequences. The superovulation regime will override the normal selection process which determines which follicles will develop fully. Exogenous gonadotrophins will also promote some aspects of oocyte maturation, but these results introduce the possibility that normal DNA methylation levels are not achieved. However, the discrepancy between the results, from each of the three experimental repeats, and the low sample numbers involved means that no conclusive answer can be reached. Additionally, it must be taken into account that the sample number within this experiment is low, particularly for the experimental repeat in which a statistically significant difference was observed (Figure 4.10B). As the power of a statistical test is proportional to the sample size of the experiment the power of this particular finding is low again adding to the limitations of this experiment. Whether the altered levels observed are due to differences between individual mice cannot be assessed as there was pooling of the superovulated oocytes in all replicates of the experiment and of the naturally ovulated oocytes obtained in the first experimental run. In follow up work it may be beneficial to keep the oocytes from each female separate to allow an assessment of whether differences in methylation level are observed between individual mice as well as increasing the number of oocytes assessed.

The observed differences in DNA methylation levels could be a consequence of post-ovulatory oocyte aging. Although ovulation is assumed to occur between 8-14 hours after the administration of hCG, the exact time period between ovulation and oocyte retrieval in either the naturally ovulating or superovulated females used in this experiment is unknown. This introduces the possibility that the variation in DNA methylation levels may actually represent aging of the oocyte rather than a hormonal effect. The naturally ovulated oocytes in the first and third experimental repeats demonstrated a trend towards higher DNA methylation levels than superovulated oocytes. It may be expected that if aging were to have any effect on DNA methylation levels, it would result in reduced global DNA methylation due to degeneration of the oocyte. However, a recent study has found that in superovulated oocytes, DNA methylation of the *Peg1/Mest* gene is completed between ovulation and fertilisation (Imamura *et al.*, 2005). This suggests that it is possible for de novo methylation to occur post-ovulation. Additionally, they found this gene underwent some demethylation in older post-ovulatory oocytes. When the current experiment was repeated, the naturally ovulated oocytes were found to have significantly lower global DNA methylation levels than the superovulated oocytes. In this case, it could either be that a longer time period had passed between ovulation and recovery of the oocytes, resulting in the loss of DNA methylation, or that the post-ovulatory de novo methylation had not yet been completed.

Even bearing in mind the widely differing results from the three repeats of this experiment the results suggest there are potential differences between the methylation status depending on whether oocytes are obtained with no stimulation or

after superovulation. It may be that the time span between ovulation and oocyte retrieval is a critical aspect of these differences. The use of an additional treatment group may help to clarify the role of post-ovulatory aging on the DNA methylation levels of oocytes. The administration of a small dose of PMSG to stimulate follicular growth used in the absence of the ovulatory dose of hCG would allow a natural ovulation to occur. Thus, in terms of post-ovulatory aging this PMSG only group would be comparable to the oocytes recovered after no stimulation. The superovulation treatment group (receiving both PMSG and hCG) would still be of interest but the timing of hCG administration and the subsequent time of ovulation would need to be carefully considered. During this experiment no assessment of the oestrous cycles of the females was attempted. By taking vaginal smears and then only placing females with the vasectomised male as they neared oestrous it may have been possible to obtain larger numbers of naturally ovulated oocytes for analysis. The addition of a control group of oocytes to allow comparison of DNA methylation levels between experimental repeats would also provide additional information as to how consistent the level of methylation of naturally ovulated oocytes were. Oocytes recovered from antral follicles were used as a potential control group. However, due to the less condensed staining pattern of these oocytes the signal intensity was too low to be captured at the same detection level as the ovulated oocytes.

Other studies that have investigated the effects of culture on genomic imprinting found that early pre-implantation embryos had altered expression of imprinted genes and aberrant methylation profiles (Doherty *et al.*, 2000; Shi & Haaf, 2002). These observations have been interpreted as being an embryonic response to the *in vitro*

environment the embryo is exposed to. However, in a large amount of these studies embryos were produced following superovulation. Thus, whether embryos were obtained by *in vitro* fertilisation or were flushed after *in vivo* fertilisation, they originated from oocytes which had been exposed to high levels of exogenous hormones. It may be that some of the observed changes to imprinting and DNA methylation in the embryo could have their origins in the superovulated oocyte, due to an altered environment during maturation.

The *fos proto-oncogene* (*c-fos*) encodes the Fos protein which is involved in regulation of transcription through activator protein-1 (AP-1) and cAMP response elements (Bakin & Curran, 1999). Fos activity is thought to be a mechanism through which extra-cellular signals can alter gene expression. DNA methylation is one of the key epigenetic mechanisms responsible for controlling gene transcription. Thus, the fact that *Dnmt1* mRNA levels increase when cells are transformed with *c-fos* may represent one of the mechanisms by which Fos influences gene expression (Bakin & Curran, 1999). This response is relatively rapid with a measurable increase in *Dnmt1* mRNA evident 4 hours after induction of *c-fos* expression (Bakin & Curran, 1999). The up-regulation of *Dnmt1* mRNA was associated with an increase in Dnmt1 protein (Bakin & Curran, 1999). Examination of the DNA methylation levels in these transformed cells found a 20% increase above control levels (Bakin & Curran, 1999). One factor that has been demonstrated to result in an up-regulation of *c-fos* in the female reproductive tract is DES, suggesting a mechanism by which oestrogenic compounds could influence DNA methylation levels (Kamiya *et al.*, 1996). The effect of the androgen, dihydrotestosterone (DHT), on *c-fos* expression in a cell line

has also been analysed and a significant increase in *c-fos* mRNA observed (Brum *et al.*, 2003). The presence of *c-fos* and Fos within the oocyte and ovary have not been extensively investigated but Fos has been localised to the porcine oocyte (Rusovici & Lavoie, 2003). A subsequent analysis of mouse ovaries did not report any Fos protein localised to the mouse oocyte, but it was found in the CL and the theca cells (Oktay & Oktay, 2004). Thus, the effect of steroids on *c-fos* expression could be a candidate mechanism by which the changes in DNA methylation observed in the current study are mediated. Further work to analyse the expression of *c-fos* in oocytes may help to elucidate the mechanisms underlying the altered DNA methylation levels observed after culture with raised steroids.

Within this study only a minority of oocytes were counterstained with PI. In retrospect PI staining of all oocytes may have allowed a more detailed interpretation of the results in this chapter. As nuclear counterstaining of all oocytes would be advantageous future work on this topic would benefit from PI staining. The images depicted in Figures 4.2 and 4.3 only underwent a visual assessment rather than full computer assisted image analysis. Initially this part of the experiment was intended as a preliminary observation to determine the scope for using the methylation immunohistochemistry. Thus, the need for a more in-depth approach to image analysis was not fully appreciated. In retrospect, the additional information that could be obtained by image analysis and PI counterstaining would be a valuable addition to this investigation and would be undertaken if the work were to be repeated. As DNA methylation levels were only assessed visually, the apparent increase in methylation from day 0 to day 6 of *in vitro* oocyte development could

represent a visual artefact resulting from the changing nuclear size and chromatin condensation. In future work this concern could be avoided by determining the ratio of mean methylation intensity to nuclear size (Beaujean *et al.*, 2004a; 2004b). With regard to making a visual assessment, merged Z-stack images would be beneficial to provide information on the 3D staining pattern. However, for any assessment of staining intensity the previously used unmerged Z-stack sections would be preferable in keeping with the image analysis undertaken in the remainder of this chapter. When the relative quantitation carried out in this chapter is considered there would have been no benefit gained from the use of merged Z-stack images. Although assessing each section separately, the image analysis method employed still ensured that the full staining information from each oocyte was obtained, analysed and incorporated into the overall results. Thus, the decision not to merge the Z-stack images does not detract from the comparative DNA methylation results presented within this study. Additionally, all the oocytes were analysed using a consistent method to ensure they were comparable. It should also be considered that the results are all presented relative to controls (relative quantitation) rather than attempting to give an absolute quantitative assessment of the level of DNA methylation present.

As there are known to be differences in reproductive parameters between individual mice all experiments were designed such that more than one female was used and the follicles/oocytes from all the animals were pooled. This means that no conclusions on variability between or within mice can be drawn from the results presented in the current study. This would perhaps be an interesting line for future investigation

where oocytes recovered from individual animals were identified within each experimental replicate.

4.5.1 Further Work

It would be of significant interest to establish whether the changes in global DNA methylation levels observed, after exposure to raised steroids, represented alteration to the methylation status of imprinted or non-imprinted sequences. The detailed analysis of specific imprinted genes methylation status could be carried out using bisulphite sequencing. Any regions which show an alteration in overall DNA methylation could be further analysed, using the methylation-sensitive single nucleotide primer extension (Ms-SNuPE) technique. This allows the methylation status of a particular cytosine residue to be analysed (Gonzalgo & Jones, 1997). However, the small number of oocytes obtained using the *in vitro* model described here would be restrictive. As can be seen in this study, the global levels of DNA vary greatly between individual oocytes. Thus, any pooling of oocytes by treatment group, as would be required to provide adequate levels for analysis, may mask any changes in methylation status present in individual oocytes.

An additional consideration when analysing actual, rather than comparative, DNA methylation levels is the protocol used. The methylation immunohistochemistry protocol requires the use of HCl to denature the DNA, as the antibody can only gain access the methyl groups on single stranded DNA. However, excess exposure to HCl can actually cause purinisation of the CpG dinucleotide resulting in artificially lowered methylation levels (Benchaib *et al.*, 2005). The exposure time was not

optimised in this study, as the rationale was to investigate methylation levels between comparable groups of oocytes. If this same approach was to be used to establish actual methylation levels, then this step would need to be optimised to ensure an accurate result.

If further analysis of the actual DNA methylation levels present were not possible, further use of the method comparing relative global DNA methylation levels would prove worthwhile. In the cultures described in this study the steroid levels were consistently high throughout the entire culture period. Further work examining the precise time at which the altered steroidal environment exerts its effects would be interesting. Ultimately, it would be important to utilise embryo transfer to investigate the long-term effects of the steroids. Although a previous study by Murray *et al.* (2005), found alterations in fertilisation rates, after steroid levels were raised during culture, the long-term implications on embryo development and survival of offspring have not been investigated.

Chapter 5

Effect of androgens and oestrogens on translocation of Dnmt1o in the oocyte

5.1 INTRODUCTION

Oocytes and pre-implantation embryos exclusively contain Dnmt1o, an N-truncated splice variant of Dnmt1 (Carlson *et al.*, 1992; Mertineit *et al.*, 1998; Ratnam *et al.* 2002). Dnmt1s has been assigned a role in maintenance methylation due to its preference for hemi-methylated DNA in biochemical assays. Since Dnmt1s adds methyl groups to the DNA during the S phase of cell division, the role of Dnmt1s as a maintenance methyltransferase depends upon cell replication. It also has significant de novo activity *in vitro* (Yoder *et al.*, 1997), thus the role for this methyltransferase *in vivo* may not be restricted to maintenance of methylation. There is conclusive evidence from knockout studies that either over-expression or loss of Dnmt1s is embryo lethal due to global genome wide hyper- or hypo-methylation respectively (Li *et al.*, 1992; Biniszkiewicz *et al.*, 2002). The inappropriate expression of *Dnmt1s*, as opposed to *Dnmt1o*, in embryos obtained by somatic cell nuclear transfer has been identified as a potential cause of the poor developmental potential observed after this experimental procedure (Chung *et al.*, 2003).

Modification of the *Dnmt1* locus such that *Dnmt1s* is expressed from the *Dnmt1o* promoter results in Dnmt1s protein being produced in the oocyte (Ratnam *et al.*, 2002). When embryos are derived from these oocytes the Dnmt1s protein undergoes the same cytoplasmic to nuclear translocation in the 8-cell embryos as Dnmt1o. Although the timing of Dnmt1s translocation is comparable to that of Dnmt1o in wildtype embryos, the cytoplasmic distribution differs (Ratnam *et al.*, 2002). The less even distribution of Dnmt1s protein may be due to differing mechanisms by which the proteins are stored and translocated. There is evidence that Dnmt1o co-

localises with annexin V in the oocyte cytoplasm, raising the possibility that this is part of the mechanism by which Dnmt1o is retained in the cytoplasm (Doherty *et al.*, 2002).

Dnmt1o is capable of substituting for Dnmt1s in embryonic stem cells suggesting that Dnmt1o has the same functional capabilities as Dnmt1s (Gaudet *et al.*, 1998). This finding is supported by a subsequent study which generated viable and fertile mice that synthesised Dnmt1o in place of Dnmt1s protein in all somatic cells (Ding & Chaillet, 2002). There could, however, be a highly specific role for the more stable Dnmt1o within the oocyte or early embryo but its function remains to be fully determined.

5.1.1 Localisation Profile of Dnmt1o

Oocytes within primordial follicles do not contain detectable levels of Dnmt1o, but in the growing oocyte high levels of Dnmt1o accumulate in the nucleus. Dnmt1o in the oocyte undergoes nuclear to cytoplasmic translocation over the later stages of oocyte development, so that in the fully mature oocyte the protein is exclusively localised to the cytoplasm (Mertineit *et al.*, 1998; Howell *et al.*, 2001). This localisation is maintained due to an active mechanism which sequesters the protein to the cytoplasm (Doherty *et al.*, 2002). Throughout almost all pre-implantation development Dnmt1o remains localised in the cytoplasm, with the exception of the 8-cell embryo when the protein again translocates to the cell nuclei (Carlson *et al.* 1992; Mertineit *et al.*, 1998; Cardoso & Leonhardt, 1999; Ratnam *et al.*, 2002).

5.1.2 Role of Dnmt1o

The identity of the methyltransferase responsible for establishing the maternal imprints in the oocyte is still debated. However, several characteristics of Dnmt1o have led to it being proposed as the enzyme responsible for maternal imprinting. The fact that the accumulation of Dnmt1o in the oocyte coincides with the period of time that maternal imprints are laid down raises the possibility of an important role for this enzyme. Over the follicular growth phase the oocyte remains in meiotic arrest. Therefore, Dnmt1o cannot be functioning in its expected role as a maintenance methylase, as this is only possible during the S phase of cell division.

The role of Dnmt1o has been investigated using *Dnmt1o*$^{-/-}$ mice, which lack Dnmt1o but have normal somatic cell levels of Dnmt1s (Howell *et al.*, 2001). When the reproductive capabilities of these mice are assessed the *Dnmt1o*$^{-/-}$ males have normal fertility levels. This was not found to be the case for females: although they did become pregnant, most embryos died between E14-21 (Howell *et al.*, 2001). The loss of Dnmt1s is embryo lethal due to genome wide hypo-methylation, but embryos from the *Dnmt1o*$^{-/-}$ females did not exhibit this global hypo-methylation (Li *et al.*, 1992; Howell *et al.*, 2001). Although global DNA methylation levels were not affected, some imprinted genes had aberrant methylation patterns resulting in inappropriate expression of these genes in the embryos. It appears that one half of the normally methylated alleles had become demethylated in the offspring of *Dnmt1o*$^{-/-}$ females. Analysis of DNA methylation patterns in the embryo does not reveal whether the aberrant methylation represents the failure of DNA methylation during oocyte development or an inappropriate loss of methylation during early pre-

implantation development. A detailed analysis of the normally maternally methylated *Snrpn* promoter found that in *Dnmt1o*$^{-/-}$ oocytes normal methylation patterns had been established, however, in the early embryos half of these alleles were unmethylated (Howell *et al.*, 2001). Further analysis of individual imprinted gene sequences in oocytes is difficult so a method allowing an assessment of genome-wide methylation status of the oocyte genome was employed. This involved replacing the maternal pronucleus of a normal zygote with that from a fertilised *Dnmt1o*$^{-/-}$ oocyte. Viable offspring were obtained from this experiment suggesting that normal maternal methylation patterns were present on the *Dnmt1o*$^{-/-}$ oocyte genome. When the reciprocal experimental transplants were carried out no viable offspring were born (Howell *et al.*, 2001). This demonstrates that the inappropriate loss of methylation from one half of the normally methylated alleles was occurring during embryonic rather than gamete development. There is conflicting evidence as to the role of Dnmt1o in methylation of the IAP sequences in the oocyte and pre-implantation embryo: Howell *et al.* (2001) found no alteration to IAP methylation in the absence of Dnmt1o expression whereas Gaudet *et al.* (2004) found Dnmt1o was required for maintenance of methylation levels at particular IAP sequences.

In the embryo, the temporary nuclear translocation of Dnmt1o has been shown to occur independently of transcription, protein synthesis and DNA replication and instead is hypothesised to be an event dependent on the period of time elapsed since fertilisation (Doherty *et al.*, 2002). This raises the possibility that the translocation of Dnmt1o observed during oocyte maturation may also occur at a pre-determined time point. As described in Section 1.6, the cytoplasmic and nuclear maturation of the

oocyte must be co-ordinated if a developmentally competent oocyte is to be produced. If the maturing oocyte is exposed to a sub-optimal environment then the synchronisation of nuclear and cytoplasmic maturation can be perturbed. It is possible that the translocation of Dnmt1o protein from the nucleus to the cytoplasm of the maturing oocyte could be one aspect of maturation which is sensitive to environmental changes. Transcripts of *Dnmt1o* are only present in the oocyte and the one cell embryo yet the protein is present in the pre-implantation embryo suggesting that the translation of the Dnmt1o protein present in the embryo occurred during oocyte development and prior to the first embryonic cleavage (Ratnam *et al.*, 2002). The fact that the full complement of embryonic *Dnmt1o* is transcribed and largely translated during oocyte development means the effects of altering the translocation of Dnmt1o during oocyte maturation could have an impact during development of the subsequent embryo.

Dnmt1o within the oocyte (if any) has yet to be conclusively determined. In addition to this there is no reason to conclude that purely because development can progress in the absence of oocyte Dnmt1o that inappropriate localisation of the protein could not prove to be detrimental to oocyte developmental competence. Even if Dnmt1o would normally have its effects during its transient nuclear localisation in the 8-cell embryo it is entirely possible that failure to undergo normal translocation processes in the oocyte could have an effect. Indeed it may be that incomplete removal of the protein from the nuclear compartment during oocyte development leaves it incorrectly positioned such that it is able to have an undesirable effect during the early stages of embryo development. Additionally, Dnmt1o interacts with Annexin

V in the oocyte (this interaction is not yet understood) thus it may be that altered translocation of Dnmt1o could have an impact on the function of Annexin V (Doherty *et al.*, 2002). It is highly desirable that the oocytes obtained by superovulation and matured or cultured *in vitro* are as physiologically "normal" as possible to allow both research work and reproductive techniques to progress. Thus investigations which help determine factors that are able to influence aspects of oocyte development are important.

5.2 AIM

The localisation of Dnmt1o is highly regulated with developmental stage in both the oocyte and early embryo. Thus, any alteration of the timing of translocation between the cytoplasm and nucleus may have an impact on the DNA methylation status and hence developmental competence of the oocyte or pre-implantation embryo. As discussed in Section 4.4.2, exposure to high concentrations of androgens and oestrogens in culture resulted in alterations to global levels of oocyte DNA methylation. Potentially, this observation could be the result of inappropriate localisation of Dnmt1o leading to aberrant methylation of the oocyte genome. The aim of this study is to investigate whether raised levels of androgens and oestrogens influence the translocation of Dnmt1o in the developing oocyte and could thereby affect global methylation levels and subsequent development.

The study required investigation of the localisation pattern of Dnmt1o after exposure to raised androgens and oestrogens in culture. However, there is no antibody available that just detects the Dnmt1o protein. Instead, the study used the PATH52 antibody (Carlson *et al.*, 1992). The epitope that the PATH52 antibody binds to is common to both Dnmt1s and Dnmt1o proteins (Carlson *et al.*, 1992; Howell *et al.*, 2001; Ratnam *et al.*, 2002). Although Dnmt1s protein is not normally found in oocytes the mRNA transcripts are present. In order to check that any Dnmt1 recognised by the PATH52 antibody was indeed due to expression of the Dnmt1o protein, a second antibody UPT82, that only detects Dnmt1s protein, was also used (Ratnam *et al.*, 2002). Antibodies were stored at 4°C in an undiluted state. Although the initial aim was to carry out an analysis of Dnmt1o after culture with all the treatment groups previously described in Table 4.1, time constraints meant that only an assessment of the A+E treatment group was able to be carried out.

5.3.1 Follicle Culture

Pre-antral follicles were dissected from 3 week old F1 ovaries and cultured for 5 days as described in Sections 2.4 and 2.5. A small number (n=21) of pre-antral follicles were not cultured; instead these were ruptured immediately after dissection and these Day 0 oocytes were then fixed for immunohistochemistry as detailed in Section 2.6.1. Cultures were carried out as previously described, in Section 2.7. For the experimental group, the basic culture medium was supplemented after filtration with Arimidex and DES, both solubilised in ETOH, to give a final concentration of 0.1μm and 4nmol respectively. Control follicles were cultured in the standard

medium with ETOH added. These groups were the same as the control and A+E treatment groups described in Table 4.1. The basic culture medium was freshly made up as required and the supplementation with steroids and ETOH was carried out immediately prior to the media being dispensed into the culture wells. The experiment was repeated on seven separate occasions with follicles obtained from a minimum of three 3 week old F1 mice being used in each replicate.

5.3.2 Dnmt1o and Dnmt1s Immunohistochemistry

Follicles were ruptured and oocytes recovered and fixed as previously described (Section 2.6.1). All steps were carried out at room temperature. Oocytes were washed in PVP/PBS (3mg/ml, Sigma-Aldrich, UK) for 15 minutes prior to permeabilisation in Triton X-100 (0.1% in PBS, Sigma-Aldrich, UK) for a further 15 minutes. The PVP/PBS wash was repeated and oocytes transferred to blocking solution (BS) for 15 minutes (0.1% BSA, 0.01% Tween 20 in PBS, Sigma-Aldrich, UK). Incubation with the PATH52 antibody (1:1000 dilution in BS; gift from T. Bestor) was carried out for 1 hour followed by two washes of 15 minutes each in BS. The secondary antibody used was donkey anti-rabbit FIT-C (Jackson Immunotech, USA) diluted 1:500 in BS for 1 hour in the dark. The final step was a 1 hour wash in BS with oocytes being mounted on positively charged Superfrost slides (VWR International Ltd, UK). After oocytes were placed on the slides excess liquid was removed prior to the addition of vectashield (Vector Laboratories, USA) and the coverslip (thickness 1, BDH, UK). Some oocytes from each treatment group (Day 0 n= 10; Control n= 32 ; A+E n= 24) were allocated to the negative control group. The same immunohistochemistry protocol was carried out on these negative controls with

the exception of the primary antibody step. Instead of incubation with the primary antibody they were transferred into a fresh BS drop.

As the PATH52 antibody described above cannot discriminate between Dnmt1s and Dnmt1o a further immunohistochemical assessment was required to demonstrate the exclusive presence of Dnmt1o. The UPT82 antibody, which only detects the Dnmt1s protein, was used to stain oocytes isolated from follicles exposed to A+E in culture (gift from R. Chaillet). All steps were carried out at room temperature. After fixing, in freshly made 4% PFA, oocytes were blocked and permeabilised for 1 hour in blocking buffer (3% BSA, 0.1% Triton X-100 in PBS). Oocytes were transferred to primary antibody, UPT82 (1:100 in blocking buffer), and left overnight at 4°C. To provide a negative control for the staining procedure a number of oocytes (n=3) were not incubated with primary antibody and were instead transferred to a fresh drop of blocking buffer. Other than the omission of the primary antibody the immunohistochemistry protocol for these negative control oocytes was normal. The following day oocytes were washed three times for 5 minutes per wash in blocking buffer before being moved to the anti-rabbit IgG alexa fluor secondary antibody (1:250 in blocking buffer; Molecular Probes, Invitrogen, UK) and left for 5 hours at 4°C in the dark. Finally three 5 minute washes in blocking buffer were carried out before oocytes were mounted on positively charged slides in vectashield mounting medium (Vector Laboratories, UK). All antibodies were stored in their undiluted state at 4°C. The PVP/PBS, Triton X-100, BSA and Tween 20 were all stored at 4°C for up to one month. The BS and blocking buffer were only made up as required and when appropriate the antibodies added immediately prior to use. After staining all

slides were stored at 4°C wrapped in foil and confocal images were obtained within 6 hours to prevent deterioration of the fluorescent signal.

All imaging was carried out using the Leica upright TCS-NT confocal microscope (Leica Microsystems UK Ltd, UK) as described in Section 2.9. The image to be obtained for analysis was the section of the oocyte in which the nucleolus was at its largest diameter as determined by a visual assessment. If no nucleolus was visible upon scanning through the entire depth of the oocyte, then the image selected for analysis was that in which the oocyte diameter was at its greatest. A single image of each oocyte at this point was captured using the confocal microscope. The negative controls were also assessed on the confocal microscope (Day 0 n= 10; Control n= 32; A+E n= 24).

5.3.3 Image Analysis

While growing oocytes have Dnmt1o in both the cytoplasm and the nucleus, the fully grown oocyte in a Graafian follicle has strong staining for Dnmt1o throughout the cytoplasm but an unstained nucleus (Mertineit *et al.*, 1998). The criteria used to assess the Dnmt1o localisation pattern in the current study were determined using these previous observations from Mertineit *et al.* (1998). The criteria used in this study are based on the hypothesis that Dnmt1o would only be able to lay down DNA methylation while localised to the GV, as once sequestered in the cytoplasm it would no longer have access to the oocyte DNA.

The staining pattern of each oocyte was assessed and categorised according to the criteria detailed in Table 5.1 below. Images from seven separate cultures were assessed using a blind analysis (Day 0 n= 11; Control n=76; A+E treatment n=72).

Table 5.1:- Description of immunohistochemistry staining categories

Group	Description of Staining
GROUP 1	Fully Stained (no GV or nucleolus visible)
GROUP 2	Fully stained GV but unstained nucleolus
GROUP 3	Partially Stained (GV still visible)
GROUP 4	Unstained GV

5.3.4 Statistical Analysis

The distribution of oocytes between each of the localisation groups after control and A+E treatment were statistically analysed using the chi-square test.

5.4.1 Dnmt1o and Dnmt1s Immunohistochemistry

To determine whether Dnmt1o was the sole variant present in the oocytes after exposure to A+E they were incubated with the UPT82 antibody. There was no positive oocyte-staining with this antibody, demonstrating that no Dnmt1s was present in the oocytes (Figures 5.1A). The cumulus cells acted as a positive control for this immunohistochemistry protocol with very strong staining evident (Figure 5.1A). All of the oocytes from Day 0 pre-antral follicles (n=11) that were stained with PATH52 had a Group 3 localisation pattern where the GV was visible but partially stained (Figure 5.1C). Several patterns of staining were observed in cultured oocytes using the PATH52 antibody (Figures 5.1D-F). There was a very small number of oocytes (n=6) which were entirely stained with no nucleolus evident (Group 1, Figure 5.1D). A higher number of oocytes had both cytoplasmic and nuclear staining while only the nucleolus remaining unstained (Group 2, Figure 5.1E). Other oocytes had some partitioning of the stain similar to that of the oocytes from uncultured follicles, such that the germinal vesicle had less staining than the cytoplasm: these were determined as having a group 3 localisation pattern (Figure 5.1F). When oocytes were analysed according to the criteria described in Table 5.1 it was found that there were no oocytes from either control or A+E cultures which had the Group 4 localisation pattern of an entirely unstained GV.

A total of 76 oocytes from control cultures and 72 from A+E supplemented cultures were analysed. The majority of control oocytes (75%) had a staining pattern consistent with Group 2, where the GV was completely stained and the nucleolus

was visible (Figure 5.2). In contrast, only 50% of oocytes exposed to A+E during the culture period had a Group 2 localisation pattern (Figure 5.2). The proportion of oocytes with partially stained GVs (Group 3) was also found to differ between the treatment groups. Only 18% of controls had a partially stained GV while 49% of oocytes exposed to A+E in culture fell into this category (Figure 5.2). A chi-square test was used to determine whether the proportion of oocytes with each localisation pattern varied between treatments, a significant difference was found (P=0.0003).

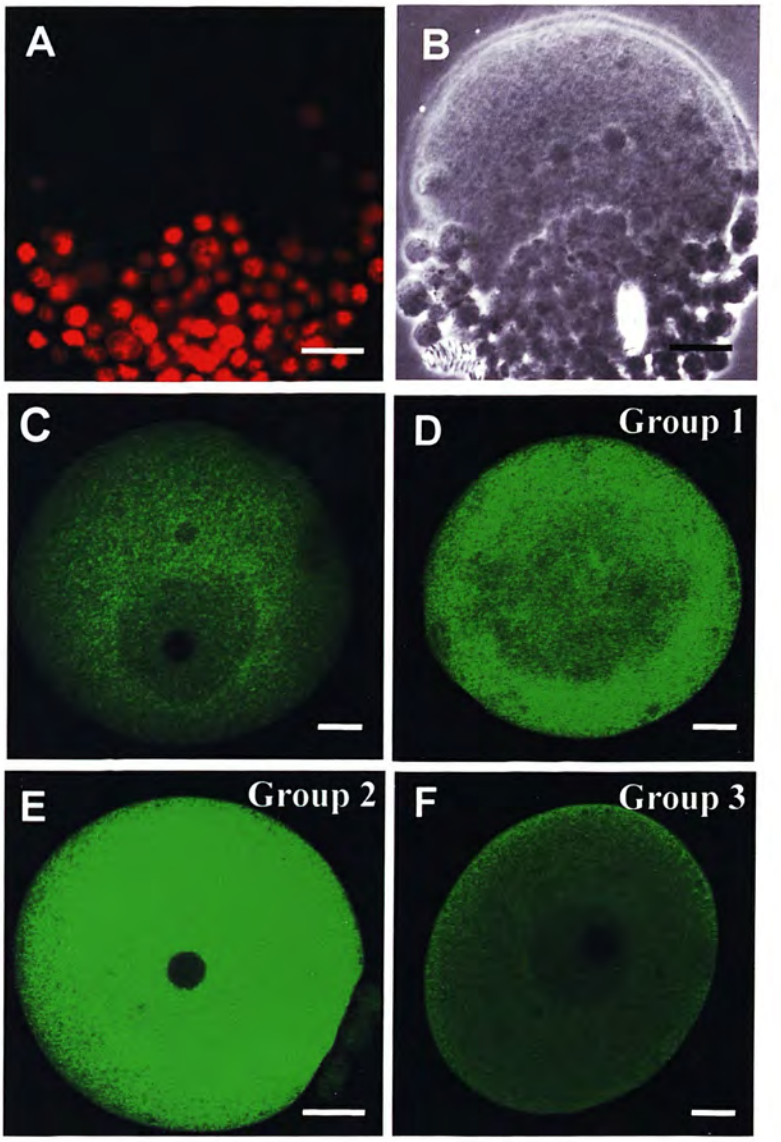


Figure 5.1:- A) Oocyte from A+E treated follicles cultured for 5 days, stained with UPT82 antibody which detects only Dnmt1s staining. The cumulus cells are strongly stained while the oocyte is unstained. **B)** Transmitted light image of the oocyte in A. **C)** Oocyte recovered from pre-antral follicle on Day 0 of culture and stained with PATH52 antibody to detect Dnmt1 (both somatic and oocyte variants) **D-E)** Oocytes cultured for 5 days and then stained with PATH52. Examples of staining **D)** Group 1, no nucleolus visible **E)** Group 2, unstained nucleolus **F)** Group 3, with GV evident All scale bars represent 10μm.

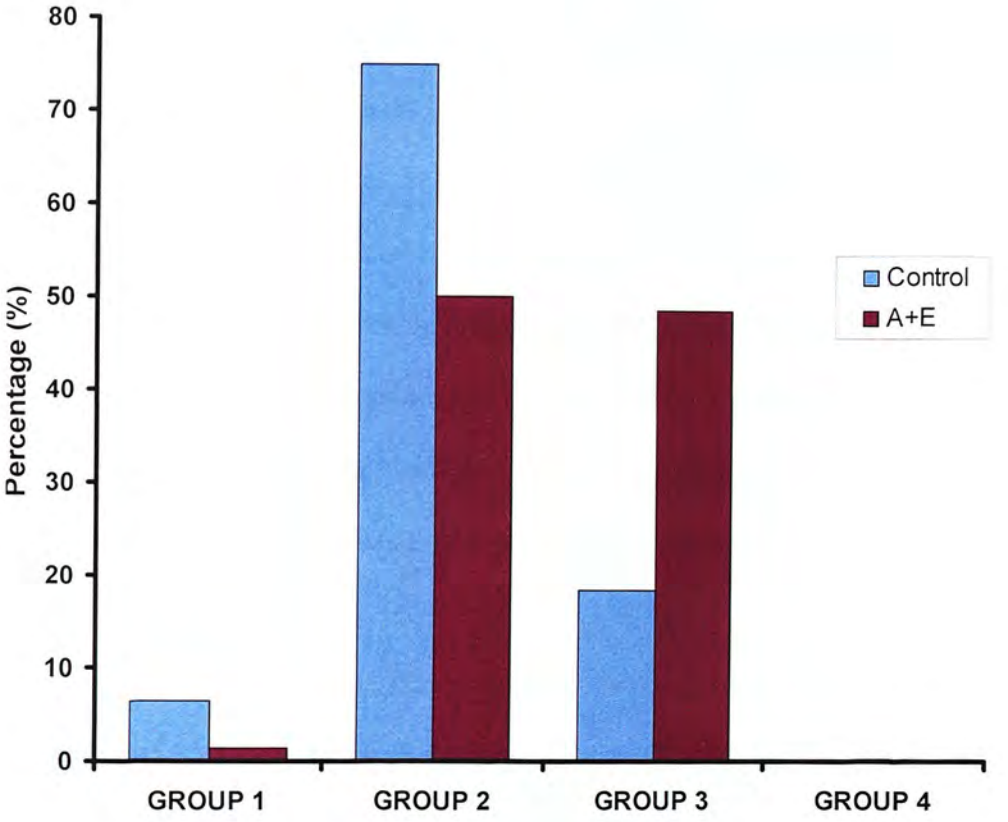


Figure 5.2:- The graph shows the percentage of oocytes in each staining pattern group according to treatment. Blue bars represent oocytes from control cultures (n=76); red bars represent oocytes from cultures where oocytes were exposed to high androgen and oestrogen in combination (A+E) (n=72). The experiment was repeated on seven separate occasions with follicles obtained from a minimum of three 3 week old F1 mice being used in each replicate. $\chi 2$ test, P=0.0003.

Group 1, no nucleolus visible
Group 2, unstained nucleolus
Group 3, with germinal vesicle evident
Group 4, unstained germinal vesicle

5.5 DISCUSSION

When oocytes from Day 5 cultures were stained with PATH52, which detects both Dnmt1s and Dnmt1o, the staining pattern obtained matched previously published observations of Dnmt1o localisation in the growing oocyte (Mertineit *et al.*, 1998; Cardoso & Leonhardt, 1999). In growing oocytes intense staining for Dnmt1o is present throughout both the nucleus and cytoplasm (Mertineit *et al.*, 1998; Cardoso & Leonhardt, 1999). To ensure that the exposure of oocytes to raised steroids did not influence which of the splice variants was translated in the oocyte, the UPT82 antibody which only detects the Dnmt1s protein was used. The results from this clearly showed that no Dnmt1s protein was present in the oocytes regardless of treatment. Thus, any change to the localisation pattern was purely due to alterations in Dnmt1o rather than aberrant translation of the somatic form.

When oocytes from pre-antral follicles recovered on Day 0 of culture were analysed they all had Dnmt1o localised to the cytoplasm with partial staining of the GV (Group 3). Inclusion of Day 0 *in vivo* oocytes was not intended as a direct comparison to the day 6 cultured oocytes. The Day 0 oocytes were stained to provide information as to the localisation of Dnmt1o prior to the culture period and to act as a positive control for the staining protocol. In growing oocytes Dnmt1o is reported to be present in both the cytoplasmic and GV compartments of the oocyte while in more mature oocytes there is an active sequestering of the Dnmt1o protein to the cytoplasm (Mertineit *et al.*, 1998; Cardoso & Leonhardt, 1999). When these previously reported dynamics of Dnmt1o translocation are considered the proportion of Day 5 oocytes with completely stained GVs are somewhat unexpected.

When comparisons were made between the staining patterns observed after culture there was a significant difference in distribution between the controls and A+E treatment group (P=0.0003). For Dnmt1o to methylate the oocyte genome it would need to be localised to the GV at the appropriate time point. When the control cultures were analysed the majority of the oocytes (75%) had a completely stained GV. In contrast, only 50% of oocytes which were exposed to high steroid levels in culture were categorised as having a completely stained GV. A further 49% have a staining pattern where the GV was visible and therefore less stained than the cytoplasm. This change in localisation suggests that the process of sequestering the Dnmt1o to the cytoplasm had been initiated in the High A+E group. This translocation of Dnmt1o protein to the cytoplasm and exclusion from the GV is correlated to increased developmental maturity of the oocyte. Thus, the oocytes exposed to raised A+E levels during culture may have accelerated maturation. As this is the group with the poor IVF result, this is, on the face of it, a surprising result. The change in Dnmt1o localisation may, however, represent impaired synchronisation of the nuclear and cytoplasmic maturation processes. As such, the previously observed reduction in developmental competence during IVF described by Murray *et al.* (2005) may be due to the precocious sequestering of Dnmt1o to the cytoplasm.

Within this study an image of a single oocyte section was assessed to determine the localisation pattern of Dnmt1o. In retrospect, the inclusion of merged Z-stack images and a nuclear counterstain could have provided further information and should be employed in future studies. It is possible that the additional information

resulting from nuclear counterstaining could have been used to pinpoint the stage of oocyte development. This may have allowed further assessment of whether the differences between the control and A+E treatment groups were due to any subtle changes to the timing of oocyte development. Although the approach to image analysis used in this study did not include Z-stack or counterstained images this does not detract from the overall findings.

When considering the results obtained in these experiments the results presented by Shi & Haaf (2002) need to be considered. One treatment that this group investigated was the addition of an ETOH derivative, acetaldehyde, to mouse embryos in culture. After exposure to acetaldehyde a large proportion of embryos were found to have abnormal DNA methylation patterns, as assessed by staining with anti-5-methylcytosine antibody (Shi & Haaf, 2002). The majority of these abnormally methylated embryos had undergone a loss of DNA methylation. This acetaldehyde treatment also affected embryo development with reduced blastocyst development reported for both the mouse strains used in the investigation, with 100% of the NMRI strain embryos failing to reach the blastocyst stage of development (Shi & Haaf, 2002). Within the follicle culture system described in this chapter the DES and Arimidex were solubilised in ETOH, this aspect of the treatment protocol could have introduced an additional factor which is capable of causing a change to the methyltransferase activity of the oocyte. However, the concentration of ETOH used in this study was only 4nmol compared to the 0.5mM of acetaldehyde added in the Shi & Haaf (2002) study. The previous study did not investigate the range of acetaldehyde doses over which the embryo DNA methylation dynamic was sensitive

to alteration. There is no conclusive evidence that the lower dose of ETOH used in the current study would be sufficiently high enough to interfere with the DNA methyltransferase activity of the oocyte. Any effect of the ETOH was controlled for in this study through the addition of 4nmol ETOH to the control culture medium. Thus, the significant difference in Dnmt1o localisation described within this chapter can be attributed to culture in the presence of raised A+E rather than simply a result of ETOH being metabolised to acetaldehyde.

A previous investigation into the translocation of Dnmt1o found co-localisation with annexin V a finding that may represent an interaction between these two proteins (Doherty *et al.*, 2002). This possibility is supported by the fact that part of the N-terminus of Dnmt1 was identified as an annexin V binding protein (Ohsawa *et al.*, 1996). As exposure to raised androgens and oestrogens in culture has a significant effect on the localisation pattern of Dnmt1o it may be that the steroids illicit this effect through alteration of the annexin V sequestering mechanism.

5.5.1 Further Work

The natural progression in this investigation would be to carry out cultures using the treatment groups in which the androgens or oestrogens are raised separately (as described in Table 4.1). This would allow more information on the role of each steroid on Dnmt1o translocation to be established.

The results described within this chapter demonstrate that there are differences in the translocation of Dnmt1o after steroid exposure. Complete disruption of the *Dnmt1o*

promoter, which prevents Dnmt1o protein accumulating in the oocyte during growth results in normal oocyte methylation levels but is embryonic lethal during later development (Howell *et al.*, 2001). It would therefore be of relevance to extend the scope of this study to investigate pre-implantation embryonic development. If changes to annexin V are involved in the altered localisation pattern of Dnmt1o observed in oocytes after exposure to raised steroids this may also have an effect in the early embryo. Dnmt1o has been shown to remain localised to the cytoplasm at all the pre-implantation stages with the exception of the 8-cell embryo, when it transiently translocates to the nucleus. The timing of this translocation is not controlled by any of the mechanisms previously investigated by Doherty *et al.* (2002) resulting in the hypothesis that translocation occurs at a time point determined by fertilisation. Annexin V is a calcium-dependent phospholipid binding protein, raising the possibility that the calcium waves occurring immediately after fertilisation could play a role in its binding ability and cellular function. If the annexin V within the oocyte is influenced by exposure to raised steroids there may be an impact on the timing of the embryonic translocation of Dnmt1o. Additionally, annexin V has been identified as an inhibitor of protein kinase C activity, one of the proteins identified as mediating formation of the second polar body after fertilisation (Shibata *et al.*, 1992; Schlaepfer *et al.*, 1992; Dubois *et al.*, 1998). Thus, further investigation of the function of annexin V within the oocyte may reveal a mechanism by which hormonal signals could impact on DNA methylation levels and developmental competence of the oocyte and early embryo.

Chapter 6

Examination of reproduction in *Mbd2*$^{-/-}$ mice

6.1 INTRODUCTION

Several members of the Mbd protein family are involved in the repression of methylated genes, as discussed in Section 1.12.3. The existence of several Mbd proteins has complicated the elucidation of their physiological functions. Mbd2 and Mbd3 are very similar proteins sharing 70% identity: the identical gene structure suggests they arose from a gene duplication event (Hendrich *et al.*, 1999). Determination of each protein's role was complicated by the *in vivo* interaction which exists between these two members of the Mbd family. In the investigation of the relationships between these two proteins gene deletion mice have been created. The *Mbd2*$^{-/-}$ animals are capable of transcribing the first two exons of the gene, the transcript of which can be translated but terminates within the methyl binding domain region (Hendrich *et al.*, 2001). The regions of the Mbd2 protein present in *Mbd2*$^{-/-}$ mice are not capable of binding to methyl groups and are lacking the regions responsible for repression as these are further downstream (Boeke *et al.*, 2000). While *Mbd3*$^{-/-}$ is embryo lethal, the *Mbd2*$^{-/-}$ mice are viable although they have some maternal behavioural abnormalities (Hendrich *et al.*, 2001).

Mbd2$^{-/-}$ mice have normal global methylation levels and normal methylation dependent silencing of both imprinted genes and IAPs (Hendrich *et al.*, 2001). Although *Mbd2*$^{-/-}$ mice appeared phenotypically normal, interesting observations as to their reproductive abilities were made. *Mbd2*$^{-/-}$ pairs had a significantly lower litter size than heterozygotes. On further investigation this reduction in litter size was found to be a trait of the female *Mbd2*$^{-/-}$ mice with the paternal genotype having no impact on litter size (Hendrich *et al.*, 2001). In addition to the reduced litter size,

there is also a reduction in the size of offspring produced by *Mbd2*$^{-/-}$ females (Hendrich *et al.*, 2001). When pups from these *Mbd2*$^{-/-}$ females were fostered onto wildtype females the weight phenotype was rescued (Hendrich *et al.*, 2001). Further investigation of the null mutant females identified problems with an important aspect of maternal behaviour; they were slower at pup retrieval. Thus, the lower pup weight seems to be due to the impaired maternal behaviour of the *Mbd2*$^{-/-}$ females (Hendrich *et al.*, 2001). Although this aberrant maternal behaviour explains the reduced weight of pups from *Mbd2*$^{-/-}$ females it does not explain the lower litter size obtained from these animals. To date, the only apparent effects of knocking out the *Mbd2* gene seem to be the reduced litter size and the behavioural defects observed. Further investigation of the maternal behavioural defects has been carried out through analysis of the imprinted *Peg1* and *Peg3* genes which are involved in nurturing behaviour. There appeared to be no defect in the expression of these or any other imprinted genes in the adult *Mbd2*$^{-/-}$ animals (Hendrich *et al.*, 2001).

To date, there has been no investigation as to the cause of the reduced litter size. The only assessment on the oocytes from these mice examined whether maternal or paternal inheritance of the null allele affected the active demethylation of the paternal genome immediately after fertilisation (Santos *et al.*, 2002): there appeared to be normal demethylation of the male pronucleus in both reciprocal crosses suggesting that Mbd2 is not an active demethylase as had previously been proposed by Bhattachrya *et al.* (1999). Thus, the influence of Mbd2 on litter size is yet to be elucidated.

6.2 AIM

The aim of this chapter is to study the effect of Mbd2 on female reproduction with regard to litter size utilising the *Mbd2*$^{-/-}$ mouse. The specific aspects which will be investigated are the number of oocytes ovulated in response to superovulatory gonadotrophins and the developmental competence of those oocytes during IVF. Analysis of these parameters will assist in the determination of whether the reduced litter size is a result of reduced ovulation number or problems which become evident during the pre-implantation stages of development. It would be hypothesised that if Mbd2 plays a role in supporting the normal development of follicles to ovulation that the *Mbd2*$^{-/-}$ females would have a reduced number of oocytes present in the oviduct after ovulation. Likewise the lack of Mbd2 could also account for the reduced litter size by reducing the developmental capacity of the pre-implantation embryo.

6.3 MATERIALS AND METHODS

6.3.1 Transgenic Mouse Colony

Originally five *Mbd2*$^{-/-}$ (C57Black/6J congenic background) pairs of mice were obtained to form the basis of a new breeding colony in the Medical Faculty Animal House (MFAA) (gift from Brian Hendrich). Although *Mbd2*$^{-/-}$ pairs formed the basis of a successful breeding colony at a previous site (Hendrich, personal communication) this was not found to be the case in the MFAA unit. At this location very few pregnancies were achieved and none of the small number of offspring survived to adulthood. To overcome this *Mbd2*$^{-/-}$ animals were paired with C57Black/6J mice with the intention of obtaining *Mbd2*$^{+/-}$ offspring which could then be used as the breeding stock for the new colony. Pups which survived to adulthood were obtained from those pairs which consisted of a C57Black/6J female with an *Mbd2*$^{-/-}$ male. The reciprocal cross only resulted in a small number of pregnancies of which few offspring survived. Adequate numbers of offspring were obtained to allow *Mbd2*$^{+/-}$/ *Mbd2*$^{+/-}$ breeding pairs to be set up. However although pregnancies were achieved the pups were of low weight and there was a high rate of death with particularly large numbers lost at weaning. Genotyping results of those offspring which survived past weaning found that almost no *Mbd2*$^{-/-}$ females were surviving. Those that did were small for their age and had a very limited response to superovulatory doses of gonadotrophins at 8 weeks of age. Only two *Mbd2*$^{-/-}$ females survived long enough to allow an assessment of ovulation at increased age and body mass. This situation continued until the MFAA unit closed. At this point the *Mbd2*$^{-/-}$ colony was re-derived in a new animal facility. *Mbd2*$^{+/-}$/ *Mbd2*$^{+/-}$

breeding pairs were set up with these re-derived animals, from which viable offspring suitable for both experimental and replacement stock were obtained.

6.3.2 Genotyping of Transgenic Mouse Colony

Ear punches were taken when pups were between 3-4 weeks of age to provide a tissue sample for genotyping. 75µl of "hot shot" alkaline lysis buffer (see below) were added to each sample and heated to 95°C for 30 minutes. Samples were cooled before 75µl of neutralising buffer (see below) were added.

"Hot Shot" Alkaline Lysis Buffer	Neutralising Buffer
25mM NaOH	40mM Tris-HCl
0.2mM EDTA pH12	

Constituents of each buffer were dissolved in water without adjustment of the pH. Buffers were stored at room temperature until required. All constituents for these buffers were obtained from Sigma-Aldrich, UK.

Primers which could distinguish between the wild type and mutant *Mbd2* alleles were used to establish the genotype of each animal. Primers (Eurogentec, Belgium) Mbd2P62 and EnP1 (see sequence below) were used to identify the mutant allele by the production of a 170bp PCR product while the 240bp product given by primers Mbd2P49 and Mbd2P61 (see sequence below) showed the presence of the wild type allele. Primers were dissolved in autoclaved distilled water to give a stock concentration of 100µM, aliquoted and frozen at -70°C to prevent freeze/thawing.

Primer Sequences (personal communication from Brian Hendrich)
KO allele – 170bp product
Mbd2P62 TTGTGGTTGTGCTCAGTTC
EnP1 TCCGCAAACTCCTATTTCTG

Wild Type allele – 240bp product
Mbd2P49 AAGAACAAGCAGAGACTCCG
Mbd2P61 ACGCTGGCCTAGTGCCGTGC

These primers were used in a multiplex PCR using the master mix shown in Table 6.1. The PCR protocol involved a denaturing step of 94°C for 10 seconds followed by annealing at 56°C for 10 seconds and an elongation step of 72°C for 40 seconds. These steps were repeated for a total of 35 cycles before a final extended elongation step of 72°C for 2 minutes was carried out. PCR products were assessed using gel electrophoresis and visualisation as described in Section 2.8.1.

Table 6.1:- Table listing the components, volume and final concentration of the PCR mix required for Mbd2 colony genotyping.

Component	Company	Volume per sample (µl)	Final Concentration
10X PCR Buffer (200mM Tris-HCl (pH 8.4), 500mM KCl)	Promega, UK	2.5	1X
MgCl2 (50mM)	Promega, UK	0.6	1.2mM
dNTP mixture (5mM each)	Promega, UK	1.0	0.2mM each
Mbd2P62 Primer (100µM)	Eurogentec, UK	0.2	0.8µM
EnP1 Primer (100µM)	Eurogentec, UK	0.2	0.8µM
Mbd2P49 Primer (100µM)	Eurogentec, UK	0.1	0.4µM
Mbd2P61 Primer (100µM)	Eurogentec, UK	0.1	0.4µM
Platinum *Taq* Polymerase (5U/µl)	Invitrogen, UK	0.2	1U
Autoclaved distilled H_2O	~	19.1	~
DNA Sample	~	1.0	~

6.3.3 Investigation of oocyte competence and embryo development of *Mbd2*$^{-/-}$ mice using IVF

Between 7-8 weeks of age the *Mbd2*$^{-/-}$ females and their *Mbd2*$^{+/+}$ female siblings were superovulated, according to the protocol described in Section 2.3. Ovaries and oviducts were removed and placed into warmed dissecting medium as described in Section 2.2 and 2.4. The ovulated oocytes were recovered from the isolated oviducts as detailed in Section 2.6.2 before being fertilised *in vitro* and the subsequent embryos cultured to the blastocyst stage (as described below in Section 6.3.3.3). If there was no *Mbd2*$^{+/+}$ female present in the litter then a *Mbd2*$^{+/-}$ sibling and an additional age-matched *Mbd2*$^{+/+}$ female were used as experimental controls. Analysis of oocyte number after superovulation was repeated four times while three experimental repeats of the IVF were carried out.

6.3.3.1 IVF

Basic stock solutions A, B, C and D for KSOM and T6 were made according to the protocol (see Appendix A) and stored at -70°C. Before use they were thawed, vortexed and spun down in a mini-centrifuge. When required both KSOM and T6 were freshly made up using the relevant basic stock solutions, according to the volumes in table 6.2 below. The final osmolarity of KSOM was 254 ± 5mOsmKg^{-1} while the T6 final osmolarity was 287 ± 5mOsmKg^{-1}. Once the osmolarity of the media were within the acceptable range the BSA was sprinkled on top of the media and left until it had dissolved. 1mgml^{-1} of BSA (Fraction V fatty acid free tissue culture grade, Sigma-Aldrich, UK) was dissolved into the KSOM while 15mgml^{-1} of BSA (Fraction V tissue culture grade, Sigma-Aldrich, UK) was dissolved into the

T6. 6 well non-tissue culture treated plastic plates (Iwaki) were used for the IVF. T6 medium was used for the sperm preparation and fertilisation drops while KSOM drops were prepared for transferring the embryos into. Media were covered with 5ml silicon oil (Merck) to prevent evaporation and the trays incubated (37°C in a humidified 5% CO_2, 95% air atmosphere incubator, Thermo Electron Corporation, UK).

Table 6.2:- Table of the required volume of each component for KSOM and T6

	Volume (ml)	
Component	KSOM	T6
A	1.0	1.0
B	1.0	1.0
C	0.1	0.1
D	0.1	0.1
Hospital Grade Water	7.8	7.8

6.3.3.2 Sperm preparation

Two 12 week old F1 male mice were killed by cervical dislocation and an abdominal incision made. The testes were removed and placed in warmed dissecting medium. The vas deferens and caudis epididymis were isolated and transferred into a drop of T6. A needle was used to apply pressure to the tube to displace the sperm and the remaining tissue then discarded leaving the sperm in the T6 medium. It was then left for 3-4 hours in the incubator to allow the sperm to undergo capacitation.

6.3.3.3 Fertilisation & Embryo Development

Females underwent superovulation as described in section 2.3 and the oocytes recovered as detailed in Sections 2.4 and 2.6.2. Oocytes were then transferred

through T6 wash drops and placed in the fertilisation drop. Sperm were added to this fertilisation drop along with the oocytes, they were then incubated (37°C in a humidified 5% CO_2, 95% air atmosphere incubator, Thermo Electron Corporation, UK) for 4-5 hours. All oocytes were moved into KSOM and incubated for a further 12 hours after which time all 2-cell embryos were moved into freshly prepared drops of KSOM, 2µl per embryo, and returned to the incubator. This system was able to support embryo development for 6 days to the hatching blastocyst stage with the number of 2-cell embryos and blastocysts being visually assessed and recorded.

6.3.3.4 Statistical Analysis

The number of oocytes retrieved after superovulation were displayed as mean ± SEM and were analysed using ANOVA. Both fertilisation rate and the percentage of 2-cell embryos developing to the blastocyst stage were analysed using the chi-squared statistical test. The F1 (C57BL/6J x CBA background) animals were acting as controls to confirm the IVF system was working rather than as experimental control group. F1 females have been used in previous IVF studies in the laboratory so the performance of their oocytes and embryos in this system are well understood and offer a way of checking the performance of the IVF protocol independently of the animals of experimental interest. Thus, the data obtained from the F1 controls were not used in the statistical analysis.

6.4.1 Investigation of oocyte competence and embryo development of *Mbd2*$^{-/-}$ mice using IVF

6.4.1.1 Original MFAA Colony

Only a single oocyte was recovered after superovulation of 8 week old *Mbd2*$^{-/-}$ females (n=2) (Figure 6.1**A**). This oocyte failed to undergo successful fertilisation, as determined by its failure to develop into a 2-cell embryo. As the *Mbd2*$^{-/-}$ females were smaller than their *Mbd2*$^{+/+}$ siblings at 8 weeks of age the experiment was repeated using 15 week old *Mbd2*$^{-/-}$ females when the size difference was less marked. After superovulation 15 ± 7 oocytes were recovered from *Mbd2*$^{+/+}$ animals while 19 ± 7 oocytes were obtained from the *Mbd2*$^{-/-}$ females (Figure 6.1**B**). Inadequate numbers of females were available for any statistical analysis to be carried out on these results.

6.4.1.2 Re-derived Colony

There was not a statistically significant difference in the number of oocytes recovered after superovulation from each genotype (Figure 6.2). Interestingly, there does appear to be a trend towards the *Mbd2*$^{-/-}$ females having an ovulation rate which is greater than *Mbd2*$^{+/+}$ animals. The mean number of oocytes for the *Mbd2*$^{+/+}$ females was 11.2 ± 2.3, while for *Mbd2*$^{-/-}$ females a mean of 14.7 ± 3.67 oocytes were recovered. The *Mbd2*$^{+/-}$ females have the smallest number of oocytes in response to superovulation, with an average ovulation number of only 6.5 ± 2.5. As these *Mbd2*$^{+/-}$ animals were only used in cases where there was no other sibling control available, only a small number underwent superovulation (n=2).

Regardless of genotype, there was no difference in the percentage of oocytes recovered which underwent fertilisation (Figure 6.3). All genotypes had a fertilisation rate between 75-84%, which compared well with the 86% fertilisation rate achieved by the F1 system controls. When the percentage of 2-cell embryos which achieved the blastocyst stage was considered there was no significant effect of genotype (Figure 6.4). However, there was a trend whereby the ability of embryos to develop to the blastocyst stage varied with maternal genotype, with embryos from both *Mbd2*$^{-/-}$ and *Mbd2*$^{+/-}$ females developing less well than those from *Mbd2*$^{+/+}$ animals.

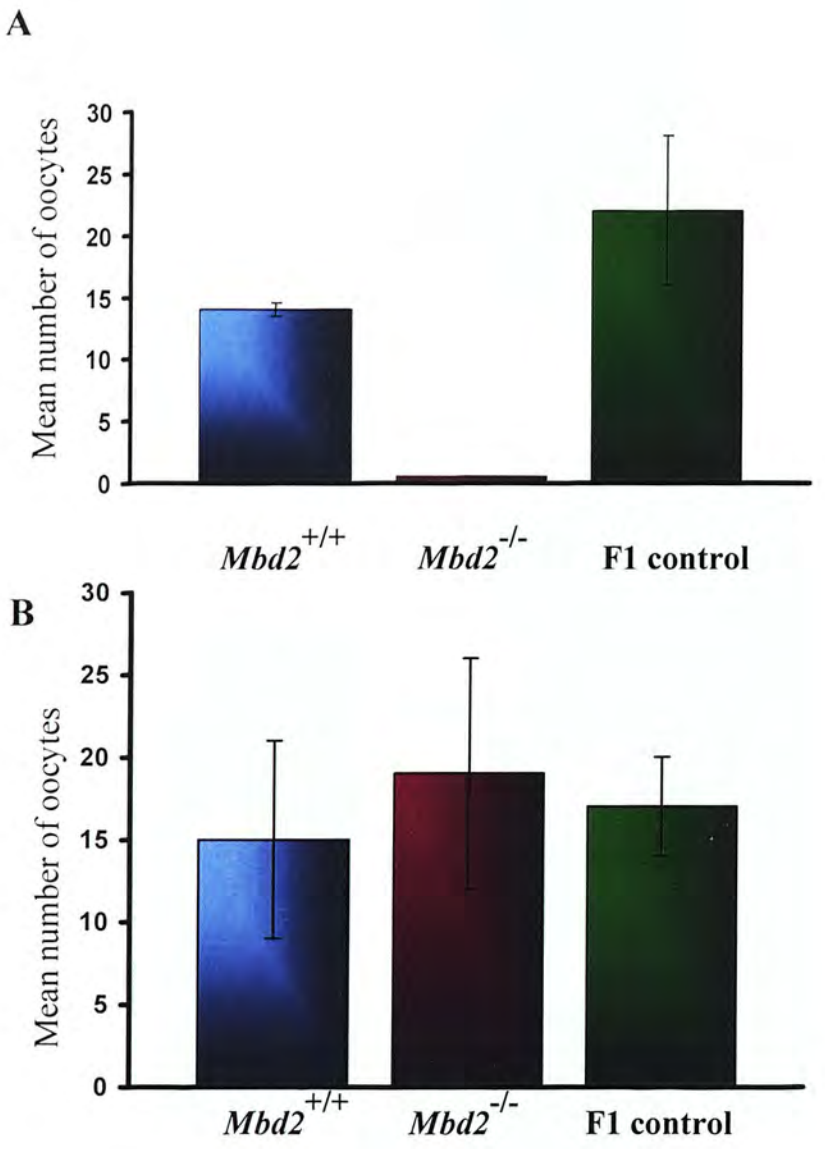


Figure 6.1:- Number of oocytes recovered after superovulation of female *Mbd2*$^{+/+}$ and *Mbd2*$^{-/-}$ offspring from original MFAA colony. Values are mean ± SEM (except *Mbd2*$^{-/-}$ in panel **A**, value given represents actual number of oocytes recovered as n=1). **A)** Graph shows the average number of oocytes recovered after superovulation, demonstrating the very poor response to exogenous gonadotrophin administration by the 8 week old *Mbd2*$^{-/-}$ (n=2), *Mbd2*$^{+/+}$ (n=2) and F1 (n=2) females. Only one run of this experiment was carried out. **B)** Graph shows the average number of oocytes recovered after superovulation of 15 week old *Mbd2*$^{-/-}$ (n=2), *Mbd2*$^{+/+}$ (n=2) and F1 (n=2) females. The data represents one experimental run.

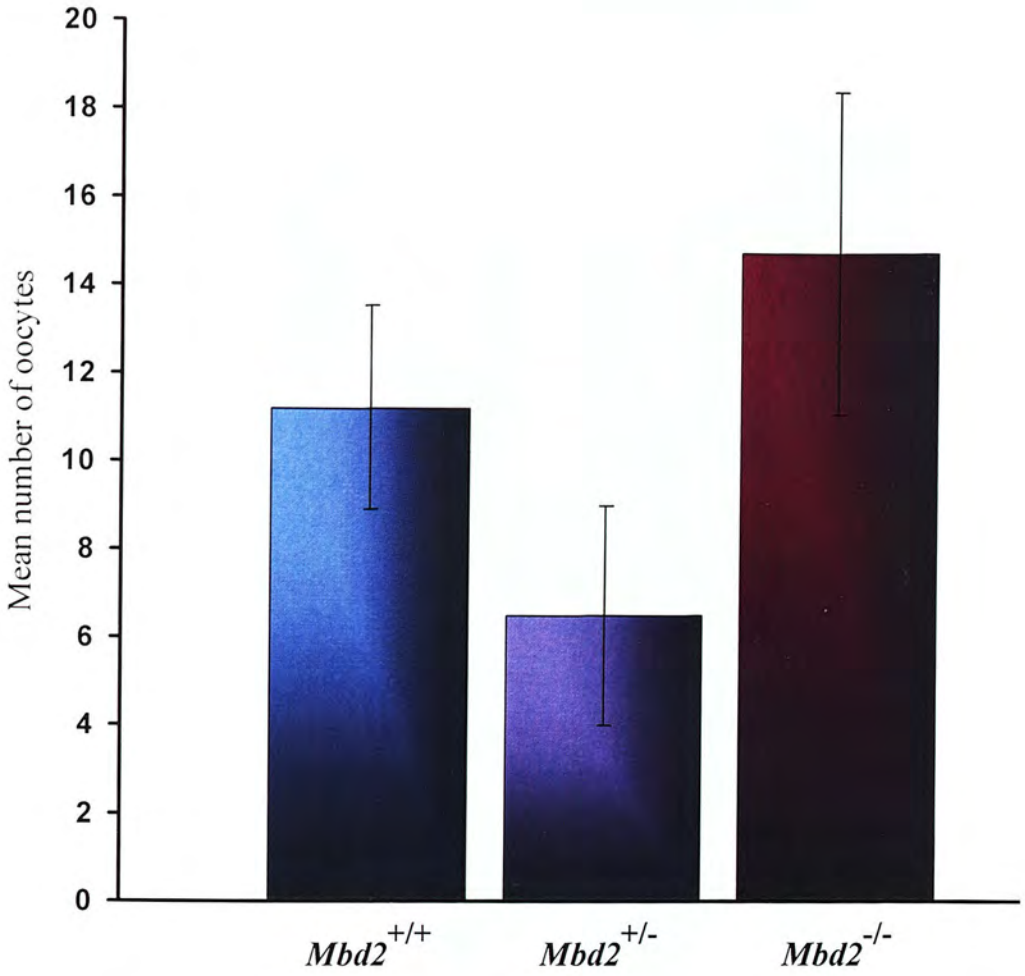


Figure 6.2:- The number of superovulated oocytes recovered by genotype. Values are mean ± SEM. There is no significant difference in oocyte number between the groups. Four experimental replicates were carried out. The number of females of each genotype used was as follows: *Mbd2*$^{-/-}$ n=8; *Mbd2*$^{+/-}$ n=2; *Mbd2*$^{+/+}$ n=9.

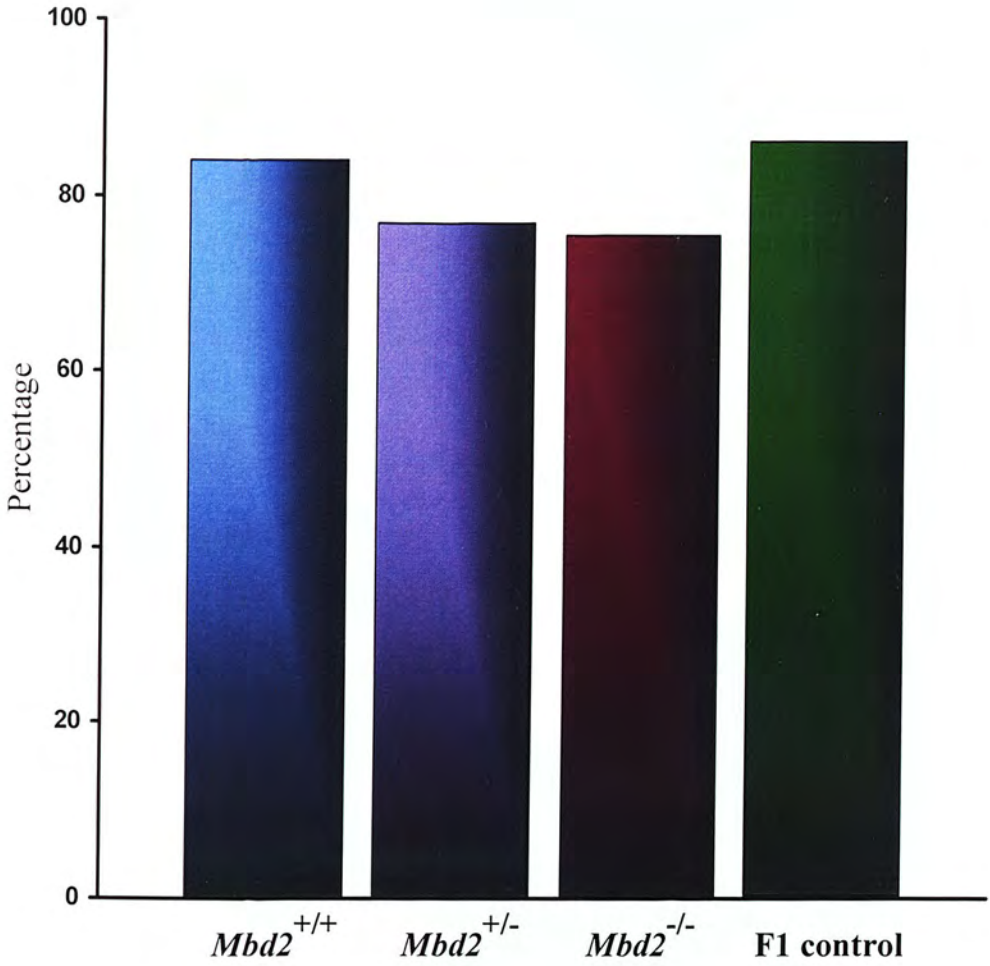


Figure 6.3:- The fertilisation rate by genotype and F1 experimental controls. The graph shows the percentage of recovered oocytes which developed into 2-cell embryos after IVF. There is no significant difference between the groups (F1 controls are not included in this statistical analysis). This experiment was repeated on three separate occasions. The number of females of each genotype used was as follows: *Mbd2*$^{-/-}$ n=7; *Mbd2*$^{+/-}$ n=2; *Mbd2*$^{+/+}$ n=7 and F1 n=9.

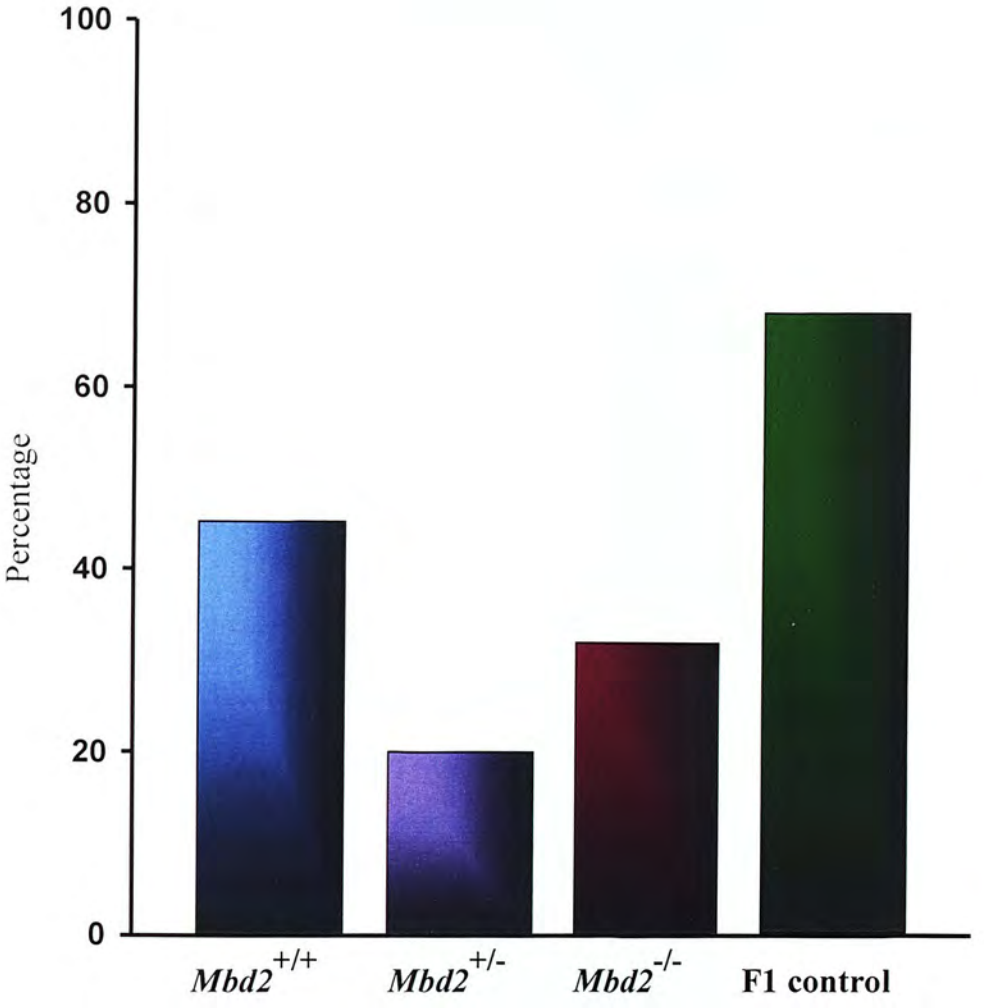


Figure 6.4:- The percentage of 2-cell embryos which developed to the blastocyst stage, according to maternal genotype. There is no significant difference between the groups (F1 controls are not included in this statistical analysis). This experiment was repeated on three separate occasions. . The number of females of each genotype used was as follows: *Mbd2*$^{-/-}$ n=7; *Mbd2*$^{+/-}$ n=2; *Mbd2*$^{+/+}$ n=7 and F1 n=9.

6.5 DISCUSSION

Although the original MFAA breeding colony did not provide enough *Mbd2*$^{-/-}$ females for a comprehensive assessment of oocyte quality and early embryo development the observed breeding problems are of relevance. The effect of the environment on the reproductive potential of the colony was considerable. Previous studies have established that environmental factors can influence epigenetic regulation of gene expression (Wolff *et al.*, 1998; Tremolizzo *et al.*, 2002; Jaenisch & Bird, 2003). This is of importance when any strain of mice are being used experimentally but when an epigenetic mechanism such as genomic imprinting and DNA methylation is being considered it is perhaps even more relevant.

The data reported here does not support an obligatory role for Mbd2 in several aspects of female reproduction. Regardless of genotype the ovulation rate, fertilisation rate and the percentage of fertilised oocytes progressing to the blastocyst stage did not alter significantly. As previously mentioned there is thought to be a certain degree of redundancy within the Mbd family of proteins (Section 1.12.3). Thus, these IVF results from the *Mbd2*$^{-/-}$ mice do not conclusively demonstrate that Mbd2 has no role in oocyte maturation or embryo development. It is possible that other Mbd proteins are able to compensate for its absence.

In addition, it should be remembered that oocyte quality, fertilisation and pre-implantation embryo development do not represent all aspects of successful reproduction. No assessment as to the ability of these embryos to implant in the uterus or their subsequent resorption rate was made in this study. As the blastocyst is

the most advanced stage of embryo development that can be supported *in vitro* this is often used as the experimental endpoint. However, the ability to progress to this developmental stage does not necessarily predict that embryos would be able to successfully implant and progress through normal post-implantation development.

As could be seen in the early breeding attempts of the original knockout colony (where the $Mbd2^{-/-}/Mbd2^{-/-}$ pairings produced almost no viable young) there was a substantial reproductive problem with these mice over and above that reported by the previous $Mbd2^{-/-}$ studies which identified a reduced litter size. Thus, this offered confirmation of a potential correlation between a lack of functional Mbd2 and poor reproductive performance. It was only when $Mbd2^{+/-}/Mbd2^{+/-}$ pairings were established in the new animal facility that adequate numbers of $Mbd2^{-/-}$ females were obtained to allow any kind of study of reproductive parameters to be assessed. Thus, investigations on unstimulated females were not carried out as the poor reproductive ability of these animals severely limited the likelihood of obtaining adequate numbers of oocytes which could subsequently be fertilised *in vitro*. The use of superovulation ensured that a maximal amount of information could be obtained from the small number of suitable females that became available during the period of study. Had more time and resources been available then a natural progression for this study would have been to assess the same reproductive parameters in unstimulated females. Although, the use of superovulatory gonadotrophins means that drawing direct comparisons between the previous studies and the results from the current study is difficult it does provide important and relevant information as to the role of Mbd2 in female reproduction. Superovulation over-rides some aspects of

the natural selection processes within the ovary allowing more follicles to develop and escape atresia. The current study shows that the lack of Mbd2 does not result in a response to superovulation that is significantly different to the control mice used in the investigation. Although there may be some aspect of follicle development or pre-implantation developmental potential that is impaired in the absence of Mbd2 and has been overcome by the high levels of exogenous gonadotrophins the study demonstrates that female murine reproduction can function normally in the absence of Mbd2.

6.5.1 Further Work

The conclusions that there are no differences in any of the fertility parameters assessed through IVF all relate to stimulated cycles. In contrast the observations that one aspect of the *Mbd2*$^{-/-}$ female phenotype is a reduced litter size was made after natural matings from unstimulated cycles. Therefore, to fully determine the effects of *Mbd2*$^{-/-}$ on female reproduction it would be important to analyse unstimulated ovulations. The same parameters which were assessed during the IVF procedure could be investigated; number of oocytes ovulated, number of 2-cell and later stage embryos flushed and an additional count of implantation sites could be carried out.

A recent paper examined the helper T-cell differentiation in *Mbd2*$^{-/-}$ mice. This study found there were differences in the cytokines produced by this transgenic mouse as regards the Th1 and Th2 profiles (Hutchins *et al.*, 2002). This could have implications for both an understanding of control of the immune system and potentially for implantation research. If implantation of the blastocyst is to

successfully occur there must be maternal immune tolerance of the allogenic fetus and placental tissues. It is thought that the presence of a particular cytokine profile consistent with a Th2 response is beneficial to developing and maintaining this immune tolerance. It may be that this is one mechanism which implantation is allowed to progress without interference from the maternal immune system. Thus, if the *in vitro* observations by Hutchins *et al.* (2002) which identified altered helper T-cell differentiation and subsequent changes to cytokine production are replicated *in vivo* it could have implications on embryo implantation. This may play a role in the reduced litter size phenotype observed in the *Mbd2*$^{-/-}$ females. If immune tolerance is not initiated although successful blastocyst development may be obtainable implantation rates may be reduced. Further examination of the *Mbd2*$^{-/-}$ females include analysis of the process of implantation and the cytokine and T-cell profile localised to the decidua could elucidate the pathways involved.

Chapter 7

General Discussion

7.1 General Discussion

The factors influencing mammalian oocyte maturation have been the subject of extensive investigations, in domestic and laboratory species as well as humans. The complex interplay of genetic and environmental factors that can affect the oocyte and subsequent embryo means that the factors which affect the process of oocyte maturation are hard to determine. Studies within this field have also been limited by the difficulty of assessing both gamete and early embryo quality, particularly in the clinical setting. Although basic morphological assessments are routinely used to determine quality, the molecular markers that characterise a developmentally competent oocyte or embryo are less well defined.

The relatively new field of genomic imprinting in reproduction and early embryo development is rapidly developing. Several recent studies have highlighted a potential correlation between the use of ART and an increase in imprinting disorders (Cox *et al.* 2002; DeBaun *et al.* 2003; Gicquel *et al.* 2003; Maher *et al.* 2003; Orstavik *et al.* 2003; Li *et al.*, 2005). These findings demonstrate the importance of understanding the processes involved in gamete maturation and embryo development. Although human oocytes and embryos can be used in experimental studies only small numbers are available and the strict legal and ethical requirements of such work limit the range of investigations that can be carried out. Due to this limited supply of human material the use of other mammalian gametes and embryos are being used to elucidate the principles of genomic imprinting, DNA methylation and the control of gene expression. The studies described within this thesis have used the murine model to study the effects of steroids on oocyte maturation. The use

of a mouse model provides several benefits over other available species. Mouse follicles can be isolated from the ovary during pre-antral follicular development and can reach the Graafian stage *in vitro*. This culture model allows the effects of specific factors on oocyte maturation to be analysed in a more controlled environment than can be achieved using *in vivo* studies. An additional benefit of utilising mice in such studies is that transgenic mice can be created to investigate the role of a particular gene and protein. Equivalent studies in the human rely on the occasional mutations that occur in disease states. Although it is known that not all data acquired using alternative mammalian species will be applicable to humans they provide a useful platform for future human studies.

Utilising a whole follicle culture system, the effects of altered steroid environment were investigated. In the presence of raised concentrations of A+E there was a significant increase in global oocyte DNA methylation levels. In contrast there was a significant reduction in DNA methylation when the oocytes were exposed to increased levels of A alone during the period of maturation. These two results had an inverse correlation to the previously described fertilisation rates of steroid treated oocytes that have been reported by our laboratory. It should also be considered that the addition of Arimidex will have the dual effect of raising androgen levels while decreasing the concentration of oestrogen due to the inhibition of the aromatase enzyme (as discussed in Section 1.15, Figure 1.10B). Thus, the two treatment groups in which a significant change to DNA methylation levels were observed both had reduced levels of endogenous oestrogens (although in the A+E group there was the addition of DES). Further assessment of DNA methylation levels after culture with

alternative preparations that alter androgen and/or oestrogen levels would allow a more comprehensive assessment of which changes to the steroidal environment were eliciting this effect. Exposure to E alone in culture did not alter the global DNA methylation levels from those seen in the control group: in contrast oocytes from this treatment group had a significantly lower fertilisation rates. Although it is possible that the altered fertilisation rates occur as a direct result of the alterations to the DNA methylation, at present it is not evident whether the change in DNA methylation levels explains the altered embryo development or if both of these are separate effects of the steroids. These observations could represent a loss of synchronisation between the maturation of the oocyte and the surrounding follicular cells. The signalling between the oocyte and the cells of the follicular compartment ensures that the oocyte undergoes the synchronised nuclear and cytoplasmic maturation which are required if the oocyte is to become developmentally competent. These alterations to DNA methylation levels could represent an effect of steroid exposure to the transcription of one of the *Dnmt* genes in the ovary. Although this possibility was investigated, the widely differing results between experimental runs did not allow any conclusion to be drawn. An investigation of *Dnmt* and *Mbd* expression in the oocyte after exposure to raised steroids would allow further analysis of this possibility.

Oocytes are routinely recovered after superovulation in the fields of both ART and research. At the time of gonadotrophin administration there will be follicles at different stages of development and atresia present in the ovaries. Thus, it would be predicted that the superovulatory hormones would have differing effects on the

follicles depending on their developmental stage. For the vast majority of developing follicles, superovulation acts to rescue those subordinate follicles which would otherwise have become atretic. Thus, an understanding of the effects of hormonal exposure on different follicular developmental stages is of consequence to ART. Within both clinical and research settings superovulated oocytes are often considered as being entirely comparable to oocytes which develop *in vivo* and undergo ovulation without induction. The results presented within Chapter 4 of this thesis suggest that there are differences in the global DNA methylation levels of oocytes recovered after superovulation and those from natural (unstimulated) ovulations. This is not to say that the one replicate demonstrating a significant difference in DNA methylation level between superovulated and naturally ovulated oocytes alone is adequate data to determine an effect of superovulation on DNA methylation levels. Rather, the fact that all three replicates of the experiment gave such different results is in itself a suggestion that perhaps superovulation has an impact on the dynamics of DNA methylation in the oocyte.

The responses observed after *in vitro* culture may not parallel the effects that would be observed after *in vivo* exposure to raised steroid levels. Although the pre-antral follicles dissected out for use in culture all appeared to be of a healthy morphology, it is possible that some of the isolated follicles were in the early stages of atresia. Late pre-antral follicles were used in these *in vitro* studies as the selection processes which determine dominant and subordinate follicles are not yet thought to be underway. However, a complete lack of follicular selection at these stages of development has yet to be conclusively proven. Bearing in mind that the early stages

of follicular atresia are not evident through a purely visual assessment it is possible that the culture conditions rescue some follicles which would otherwise have been destined to die through atresia. However, the effects of any molecular changes occurring during early selection and atresia may not be entirely reversible. Taking this into account it is plausible that some of the oocytes in each treatment group would in fact have been retrieved from follicles that had been rescued from the atretic pathway. It may be the case that these follicles and their oocytes would respond differently when exposed to raised steroid levels in culture than those in which no atretic signalling pathways had been initiated.

When Dnmt1o localisation patterns were investigated, a greater proportion of the A+E treated oocytes were assessed as having reduced GV localisation of the protein than was seen in the Control group. While the majority of the Control oocytes had a completely stained GV, many of the A+E oocytes had only a partially stained GV. A simple interpretation of this difference in localisation pattern would be that the A+E oocytes would have reduced DNA methylation levels due to a reduction in the level of the methylase enzyme present in the GV. However, when A+E treated oocytes were assessed they were found to have a significantly higher level of DNA methylation than the controls. Initially, these two results may appear to be contradictory but on further consideration this may not be the case. The analysis of the Dnmt1o localisation pattern reported in Chapter 5 was carried out at only one time point during oocyte maturation. The altered localisation pattern may in fact represent an accelerated translocation of the Dnmt1o protein, as opposed to a failure of the protein to become localised to the GV. Further information on the effect of

steroids on the localisation of Dnmt1o could be obtained by undertaking a time-course experiment. It should also be borne in mind that the actual role of Dnmt1o within the oocyte has yet to be elucidated. There is conflicting data as to whether it plays a role in DNA methylation during oocyte maturation or acts exclusively during the 8-cell stage of embryo development.

The investigation into the small litter phenotype of the *Mbd2*$^{-/-}$ female mice did not yield any significant results when ovulation number, fertilisation rate or blastocyst development were assessed. These results could be interpreted as demonstrating that the Mbd2 protein does not play a role in these reproductive processes and that the phenotype of small litter size is the result of a separate effect of Mbd2, perhaps on implantation. It could, however, be that the loss of Mbd2 does have a negative impact on one of the investigated parameters but that this effect can be compensated for by the administration of exogenous superovulatory gonadotrophins.

7.2 Future Work

As well as the laying down of DNA methylation patterns in the gametes, the appropriate demethylation of the parental genomes after fertilisation is required for normal embryo development to proceed. There are two steps involved in this process, the rapid, active demethylation of the paternal genome immediately after fertilisation and the slower, passive demethylation of the maternal genome. Work by Beaujean *et al.* (2004c) established that the active process of demethylation is controlled primarily by the oocyte. Thus, it is plausible that impaired oocyte

maturation may impair the capability of the oocyte cytoplasm to exert this demethylating activity. It would be worthwhile to investigate whether steroids affect the oocyte in a way which impairs its ability to demethylate the paternal genome. In conjunction with this analysis of paternal demethylation, the remodelling of the chromatin should also be analysed as these two processes are linked. The process of passive demethylation is also important with the DNA methylation levels of embryos obtained as a result of somatic cell nuclear transfer being aberrantly high, potentially through a reduction in the amount of passive demethylation occurring (Dean *et al.*, 2001). Oocytes obtained from follicles exposed to the culture conditions described in Section 4. 3.3 could be subjected to IVF and then fixed. The assessment of the DNA methylation levels of both the maternal and paternal genome at several time points after fertilisation would allow both the initial process of active demethylation and the later passive demethylation of the maternal genome to be evaluated.

An important progression of this study would be to carry out embryo transfers to see whether the exposure to androgens and/or oestrogens during oocyte maturation had any impact later in development. As not all modifications to the oocyte or early embryo result in immediate failure of development more long-term analysis of the embryo and subsequent offspring is desirable. Although alterations to global DNA methylation levels were identified in this study it may be that abnormally hyper- or hypo-methylated oocytes are capable of normal development after fertilisation. There may be mechanisms within the oocyte or early embryo which allow correction of DNA methylation levels. Alternatively, the normal process of demethylation and reprogramming that occur after fertilisation may compensate for the altered DNA

methylation levels observed. During early pre-implantation development there is demethylation of repeat regions and other non-imprinted sequences, while the imprinted genes are protected and maintain their methylation patterns. The fact that previous IVF data after steroid exposure found that the ability of the embryos to reach the blastocyst stage was not affected may suggest that the altered methylation is not occurring at the imprinted genes but in the non-imprinted sequences which ordinarily undergo epigenetic reprogramming, and are therefore reset, during early stages of embryo development.

7.3 Clinical Impact

The rate of pregnancy success after ART is currently stalled at approximately 20-25%. Any investigations which lead to an improved understanding of oocyte quality and the factors which have an impact on developmental competence of gametes or pre-implantation embryos could be beneficial to the field of assisted reproduction. Oocytes used in ARTs are exposed to an altered hormonal environment due to the administration of exogenous gonadotrophins, which will in turn cause an increase in intra-follicular androgens and oestrogens. Evidence presented within this thesis demonstrates that exposure to raised steroids can influence the DNA methylation levels of the oocyte genome. As correct DNA methylation is vital for normal gene expression and embryo development it is possible that impaired methylation is a contributing factor in the low rate of pregnancy achieved after ART. Improvements in ARTs could increase the success rates and the chances of a "take-home" baby resulting from each cycle. Additionally, an understanding of the mechanisms involved in developmental competence could ultimately allow only one embryo to be transferred, thus reducing the level of multiple pregnancies resulting from ART. This is of particular interest in the clinical field as a multiple pregnancy is associated with higher risks for both mother and baby.

7.4 Conclusion

One of the main limits on research in this field is a lack of knowledge on what constitutes a "normal" oocyte. Many studies utilise superovulated oocytes in their research but this thesis raises questions as to how physiological the DNA methylation status of these oocytes actually is. The results presented within this thesis add support to the hypothesis that the presence of androgens and oestrogens at higher than physiological concentrations have an impact on oocyte maturation. This study is the first to demonstrate that global DNA methylation levels of oocytes can be altered by exposure to raised steroid levels. There is an increasing body of evidence supporting the concept that altered environmental conditions can have an impact on oocyte quality and the subsequent development of an embryo. However, the specific environmental factors and the mechanisms through which they exert their effects are unclear. This thesis demonstrates a correlation between altered steroidal exposure of the oocyte and changes to DNA methylation levels during oocyte maturation, potentially through altered Dnmt1o localisation. The implications of this finding are far reaching, both in the clinical and research setting.

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Appendices

APPENDIX A: - IVF Solutions

IVF Stock Solutions

Solution A

KSOM constituents 100ml of x10 stock	g $100ml^{-1}$	Final Conc. (mM)
NaCl	5.550	95.0
KCl	0.186	2.5
KH_2PO_4	0.048	0.35
$MgSO_4$ $7H_2O$	0.049	0.20
Na lactate (60% syrup)	1.869	10.0
Glucose	0.036	0.2
EDTA	0.004	0.01

T6 constituents 100ml of x10 stock	g $100ml^{-1}$	Final Conc. (mM)
NaCl	5.719	97.84
KCl	0.106	1.42
$MgCl_2$ $6H_2O$	0.096	0.47
Na_2HPO_4 $12H_2O$	0.129	0.36
Na lactate (60% syrup)	4.652	24.90
Glucose	1.000	5.56

Solution B

Common to both **KSOM & T6 constituents** 100ml of x10 stock	g $100ml^{-1}$	Final Conc. (mM)
$NaHCO_3$	2.101	25.00
Phenol Red	0.010	

Solution C

KSOM constituents 100ml of x10 stock	g $100ml^{-1}$	Final Conc. (mM)
Na pyruvate	0.022	0.2

T6 constituents 100ml of x10 stock	g $100ml^{-1}$	Final Conc. (mM)
Na pyruvate	0.055	0.47

Solution D

Common to both **KSOM & T6 constituents** 100ml of x10 stock	g $100ml^{-1}$	Final Conc. (mM)
CaCl2 2H2O	0.252	1.71

Stocks of A, B, C and E were stored at -70°C for 6 months while stocks of D were stored at -70°C for 3 months.

APPENDIX B: - COMPANY ADDRESSES

Acumedic Limited
101-105 Camden High Street
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Alpha Innotech Corporation
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Bioquell UK Limited
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Bio-Rad Laboratories Ltd.
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Bitplane AG
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Wilford Industrial Estate
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Intervet UK Limited
Walton Manor
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UK

Vector Laboratories Ltd.
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PRESENTED WORK

APPENDIX C: - NOW2004, Edinburgh, 2004

Platform Presentation, Awarded Session Prize

Mouse oocytes exposed to raised androgen and oestrogen levels *in vitro* exhibit increased DNA methylation.

Swales A.K.E. & Spears N.

SBCLS, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh, EH8 9XD, UK.

If normal embryo development is to progress after fertilisation, the oocyte must have undergone coordinated nuclear and cytoplasmic maturation. As part of this maturation it is vital that epigenetic reprogramming occurs, involving the addition of methyl groups to the DNA of imprinted genes. Studies have found that errors in imprinting are commonly seen after oocytes or embryos have been exposed to a period of time in culture, which can result in disorders such as large offspring syndrome. Although effects on imprinting have been identified, the aspects of the culture system responsible have yet to be identified. Further studies in this area could be beneficial for both Assisted Reproductive Techniques and cloning. Preantral follicles were cultured in either control media or media with raised levels of androgens and oestrogens. Steroid levels were altered using Arimidex (an aromatase inhibitor which will increase androgens by preventing their conversion to oestrogen) and Diethylstilboestrol (DES, a synthetic oestrogen). Previous unpublished results from this lab demonstrated impaired developmental competence of oocytes cultured using this regime. After 5 days in culture, follicles were ruptured and the oocytes recovered. Methyl groups on the oocyte DNA were stained using immunocytochemistry: images were obtained using a confocal microscope and then analysed. Oocytes exposed to raised levels of androgens and oestrogens during the culture period had a significantly raised level of global methylation compared to controls (P=0.0015). The increase in global methylation identified could represent incorrect methylation of imprinted genes. This result proves that an *in vitro* environment high in both androgens and oestrogens can affect the maturation of the oocyte.

Acknowledgements: Thanks to Nathalie Beaujean for her time and advice and Linda Wilson for help with the confocal work and image analysis.

APPENDIX D: - XIth Ovarian Workshop, Vancouver, 2004

Title of poster

Mouse oocytes exposed to raised androgen and oestrogen levels *in vitro* exhibit increased DNA methylation.

Objective

If normal embryo development is to progress after fertilisation, the oocyte must have undergone coordinated nuclear and cytoplasmic maturation. As part of this maturation it is vital that epigenetic reprogramming occurs, involving the addition of methyl groups to the DNA of imprinted genes. Studies have found that errors in imprinting are commonly seen after oocytes or embryos have been exposed to a period of time in culture, which can result in disorders such as large offspring syndrome. Although effects on imprinting have been identified, the aspects of the culture system responsible have yet to be identified. The aim of this study was to investigate the effect of raised androgen and oestrogen levels *in vitro* on oocyte methylation levels.

Design

Materials/Methods

Preantral follicles were cultured in either control media or media with raised level of androgens and oestrogens. Steroid levels were altered using Arimidex (an aromatase inhibitor which will increase androgens by preventing the conversion of androgen to oestrogen) and Diethylstilboestrol (DES, a synthetic oestrogen). After 5 days in culture, follicles were ruptured and the oocytes recovered. Methyl groups on the oocyte DNA were stained using the 5-methyl cytosine antibody in immunocytochemistry: images were obtained using a confocal microscope and then analysed.

Results

Oocytes exposed to raised levels of androgens and oestrogens during the culture period had significantly increased global methylation compared to controls ($P<0.0001$).

Conclusions

The increase in global methylation identified could represent incorrect methylation of imprinted genes. This result proves that an *in vitro* environment high in both androgens and oestrogens can affect the development of the oocyte. Further studies in this area could be of significance to Assisted Reproductive Techniques.

Supported by
BBSRC

APPENDIX E:- SSR Abstract, Vancouver, 2004

Platform Oral Presentation

MOUSE OOCYTES EXPOSED TO RAISED ANDROGEN AND OESTROGEN LEVELS *IN VITRO* EXHIBIT INCREASED DNA METHYLATION.

Swales A.K.E. & Spears N.

University of Edinburgh, Edinburgh, UK.

If normal embryo development is to progress after fertilisation, the oocyte must have undergone coordinated nuclear and cytoplasmic maturation. As part of this maturation it is vital that epigenetic reprogramming occurs, involving the addition of methyl groups to the DNA of imprinted genes. Studies have found that errors in imprinting are commonly seen after oocytes or embryos have been exposed to a period of time in culture, which can result in disorders such as large offspring syndrome. Although effects on imprinting have been identified, the aspects of the culture system responsible have yet to be identified. Further studies in this area could be beneficial for both Assisted Reproductive Techniques (ARTs) and cloning. Preantral follicles were cultured in either control media or media with raised level of androgens and oestrogens. Steroid levels were altered using Arimidex (an aromatase inhibitor which will increase androgens by preventing the conversion of androgen to oestrogen) and Diethylstilboestrol (DES, a synthetic oestrogen). Previous unpublished results from this lab demonstrated impaired developmental competence of oocytes cultured using this regime. After 5 days in culture, follicles were ruptured and the oocytes recovered. Methyl groups on the oocyte DNA were stained using the 5-methyl cytosine antibody in immunocytochemistry: images were obtained using a confocal microscope and then analysed. Oocytes exposed to raised levels of androgens and oestrogens during the culture period had significantly increased global methylation compared to controls ($P<0.0001$). The increase in global methylation identified could represent incorrect methylation of imprinted genes. This result proves that an *in vitro* environment high in both androgens and oestrogens can affect the maturation of the oocyte.

Appendix F & G

MANUSCRIPTS

REVIEW

Genomic imprinting and reproduction

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Abstract

Genomic imprinting is the parent-of-origin specific gene expression which is a vital mechanism through both development and adult life. One of the key elements of the imprinting mechanism is DNA methylation, controlled by DNA methyltransferase enzymes. Germ cells undergo reprogramming to ensure that sex-specific genomic imprinting is initiated, thus allowing normal embryo development to progress after fertilisation. In some cases, errors in genomic imprinting are embryo lethal while in others they lead to developmental disorders and disease. Recent studies have suggested a link between the use of assisted reproductive techniques and an increase in normally rare imprinting disorders. A greater understanding of the mechanisms of genomic imprinting and the factors that influence them are important in assessing the safety of these techniques.

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What is genomic imprinting?

Genomic imprinting is the parent-of-origin specific gene expression and is determined by epigenetic modification of genes, such that gene transcription is altered while the actual gene sequence remains unchanged. Genomic imprinting results in only one inherited copy of the relevant imprinted gene being expressed in an embryo. For paternally imprinted genes, the paternal allele is epigenetically modified to prevent transcription, ensuring that the embryo has only mono-allelic expression from the maternally inherited copy. The opposite is true of maternally imprinted genes, when only the copy inherited from the father is expressed. The fact that particular genes are differentially expressed, according to their parent-of-origin, means that during development the parental genomes are functionally non-equivalent (Surani 1998). Genomic imprinting is vital for normal gene expression patterns in an individual, with errors sometimes resulting in inappropriate gene transcription or repression. Within the mouse genome, approximately 80 imprinted genes have so far been identified (Beechey *et al.* 2005). It is likely that there will be a similar number of imprinted genes in humans, although fewer have been found to date. Except where stated, this review refers to work on the mouse, as this species has been by far the most extensively studied species.

Within the mammalian genome, the majority of cytosine residues found as CpG dinucleotides (i.e. those cytosines positioned next to a guanine residue) have a methyl group added to their carbon 5 position (Costello & Plass 2001). It is this addition of the methyl group, referred to as DNA methylation, that is proposed as the key mechanism (certainly the best studied mechanism) regulating imprinting. It is important to point out, however, that the majority of methylated DNA in the genome is not concerned with genomic imprinting. Heavy methylation of DNA results in a more condensed structure which is resistant to transcription. Thus, if an animal inherits a methylated copy of a gene from its mother and a non-methylated copy from its father, the maternal copy will have its transcription repressed leaving the paternal copy as the only active gene. DNA methylation is an epigenetic modification which can be inherited in a stable manner but is also reversible, allowing gender specific patterns to be initiated in germ cells.

Why did genomic imprinting evolve?

The most commonly proposed theory explaining the development of genomic imprinting is the genetic conflict or parental investment theory (Moore & Haig 1991). This theory arose from the observation that many imprinted genes are implicated in the growth and development of the mammalian fetus or placenta. In addition to this, imprinted genes have been shown to exhibit directionality in their actions; that is the majority of the paternally expressed genes, such as *Igf2* and *Peg3*, promote fetal growth and nutrient uptake while in contrast the maternally expressed genes, such as *Igf2R* and *Gnas*, tend to curb fetal growth (Reik & Dean 2001, Tycko & Morison 2002). Females who could restrict fetal growth and

ISSN 1470–1626 (paper) 1741–7899 (online)

DOI: 10.1530/rep.1.00395
Online version via www.reproduction-online.org

produce more offspring from their limited resources would, in the long term, be more successful. In contrast, males would derive long-term benefit from their progeny being larger and stronger even if they achieved this to the detriment of the mother by utilising more maternal nutrients.

An alternative theory to account for the presence of imprinted genes within the genome is the evolvability model (Beaudet & Jiang 2002). This predicts that species which have genomic imprinting are more able to evolve in response to environmental pressures, as they can induce rapid changes by altering which of the two alleles is silenced and which is expressed. An individual organism can carry an allele which promotes growth that, while imprinted, has no phenotypic effects. Thus, if increased growth becomes advantageous, the relevant allele is already present in the gene pool: by rapid reversal of the imprinting, the allele can be expressed. There is also the 'ovarian time bomb' concept which proposes that genomic imprinting evolved to protect the female from ovarian disease: Varmuza & Mann (1994) hypothesised that imprinting could limit the level of growth and development of any parthenogenetic embryos within the ovary, thereby preventing malignant trophoblast formation.

How are DNA methylation patterns regulated?

DNA methyltransferases (Dnmts) carry out methylation of DNA; these can be broadly divided into Dnmt1, Dnmt2 and Dnmt3 families. The three families of Dnmts are related, albeit distantly, and are believed to have diverged from their common ancestors prior to the separation of the animal and plant kingdoms (Howell *et al.* 2001). Members of both the Dnmt1 and Dnmt3 families have been identified as having active transmethylase activity and their functions have been partially elucidated, with the Dnmt3 family being primarily concerned with laying down new methylation patterns while the Dnmt1 family appears to be mainly involved in the maintenance of these patterns during cell division. Initial studies on Dnmt2 did not find any active methylation function for this protein but more recent research has challenged this concept, with work by several groups finding that this enzyme can act as a methyltransferase which targets a very specific DNA sequence, explaining the low level of identifiable activity (Liu *et al.* 2003, Hermann et al. 2003, Tang *et al.* 2003). Although capable of binding to methylated DNA, the definitive binding specificity of Dnmt2 has yet to be determined (Hermann et al. 2003). Golding & Westhusin (2003) have shown that Dnmt2 is actually the most prevalent Dnmt in the bovine adult ovary and testis.

Laying down of methylation patterns

To allow reprogramming of the germ cell, the genome must undergo demethylation (as described later). Once the initial imprints have been removed the appropriate new pattern must be established, thus ensuring that the paternal- and maternal-specific imprints are laid down in the sperm and oocyte respectively. The enzymes which are capable of laying down the new methyl groups onto previously unmethylated DNA are from the Dnmt3 family. Members of this family which have active transmethylase activity are Dnmt3a and Dnmt3b, which share a high degree of sequence homology but have been shown to have different expression patterns and timing through development. The third member of this family, Dnmt3l, shares sequence homology with the other enzymes but is missing the catalytic domain needed to add methyl groups onto DNA. After both examining the localisation of this protein and using mice with a disrupted *Dnmt3l* gene, a role in the establishment of maternal imprints in the oocyte has been hypothesised for this enzyme, as discussed below.

Maintenance of methylation

When a methyl group is added onto already hemimethylated DNA during cell replication (necessary if the daughter cells are to maintain the methylation pattern of the cell undergoing mitosis), the process is termed maintenance methylation. Dnmt1 has the primary responsibility for maintaining the methylation status of DNA. The most common form of this methyltransferase is that found in all somatic cells, Dnmt1s, and has been shown to be vital for development. In addition, there are two splice variants identified which are specific to the germ cells and early embryo. Dnmt1p is found in pachytene spermatocytes whilst Dnmt1o is only identifiable in the oocyte and pre-implantation embryo. It is not until embryonic day 7 (E7) that the embryo is capable of producing full-length Dnmt1s protein.

Interestingly, although Dnmt1 has been identified as the main maintenance methylase *in vivo*, studies *in vitro* have shown that this enzyme has a higher *de novo* methylase activity than either Dnmt3a or Dnmt3b. *In vivo*, Dnmt1 *de novo* methylase activity has yet to be found, but the possible implications of this *in vitro* activity should be borne in mind (Howell *et al.* 2001).

How does methylation lead to repressed gene transcription?

There are two main mechanisms by which the methylation of DNA can prevent the transcription of genes. The first of these is by the methyl group causing direct interference preventing particular transcription factors from binding to methylated DNA (Iguchi-Ariga & Schaffner 1989). The second mechanism results from methyl-binding domain proteins (MBDs) binding to methylated DNA.

Of the MBDs identified to date, MBD1 to MBD3 and methyl CpG-binding protein 2 (MeCP2) are involved in transcriptional repression (Nan *et al.* 1997, Fujita *et al.* 1999, Ng *et al.* 1999), while MBD4 is thought to have a role as a mismatch repair protein (Hendrich *et al.* 1999).

MBD1 and MeCP2 both contain transcriptional repression domains which act via histone deacetylases (HDACs). HDACs cause local deacetylation of the histone tails which, in turn, results in remodelling of the chromatin into a more condensed structure that is resistant to transcription (Taunton *et al.* 1996). MBD1 mediates transcriptional repression through recruitment of a histone methylase capable of binding HDACs (Ng *et al.* 2000, Fujita *et al.* 2003), while MeCP2 acts to bind a co-repressor complex containing an HDAC (Jones *et al.* 1998, Nan *et al.* 1998), although MeCP2 has also been shown to cause transcription repression in the absence of HDAC activity (Nan *et al.* 1998, Yu *et al.* 2000). MBD2 and MBD3 are both components of a large protein complex, MeCP1 (Feng & Zhang 2001). MeCP1 binds methylated DNA in a non-sequence-specific manner. The binding of MeCP1 to methylated DNA is due to the presence of MBD2 in the complex (Ng *et al.* 1999). Interestingly, the mammalian form of MBD3 appears not to bind directly to methylated DNA (Hendrich & Bird 1998). The MeCP1 complex binds methylated DNA less tightly than MeCP2, which suggests that long-term transcriptional repression may be maintained by the permanent binding of MeCP2, with more transient transcriptional silencing determined by the binding of the MeCP1 complex (Ng *et al.* 1999). In addition to the MBD family, there is a further binding protein termed Kaiso which is capable of methylation-dependent repression of gene transcription. Although it is not an MBD-containing protein, it is capable of binding to methylated DNA via its zinc finger (Prokhortchouk *et al.* 2001). Kaiso has been shown to be a vital component of amphibian development; blocking translation of this protein is lethal (Ruzov *et al.* 2004) but the extent of its role in mammalian systems has yet to be established. Methylation-dependent transcriptional repression is covered by many good reviews such as Wade (2001) and Li (2002).

Transgenic studies show that mice lacking MBD1 have no observable phenotype, although problems within the nervous system are evident at the molecular level (Zhao *et al.* 2003). *Mbd2* knockout (KO) mice are also viable, although they exhibit impaired maternal behaviour (Hendrich *et al.* 2001). *Mbd3* null mutations are embryo lethal (Hendrich *et al.* 2001). The abnormal phenotype of *Mecp2* KO mice develops from several weeks of age and is lethal by 8 weeks of age, with all known abnormalities having their origin in the nervous system (Guy *et al.* 2001). The fact that *Mbd1*, *Mbd2* and *Mbd3* KO mice have no apparent phenotype outside of the nervous system suggests that there is a degree of redundancy within the MBD-mediated system of transcription control. Although a double KO of *Mbd2* and *Mecp2* has demonstrated that both these proteins function in separate pathways, this does not rule out co-operation between other members of the MBD family (Guy *et al.* 2001).

Genomic imprinting in germ cells and embryos

Primordial germ cells

When primordial germ cells (PGCs) are first seen in the mouse embryo at E7 they, and the surrounding somatic cells, carry the maternally and paternally inherited imprinting patterns. This DNA methylation pattern is maintained in PGCs as they migrate to the developing gonad. Coincident with their arrival in the gonadal ridge, the mouse PGCs begin to undergo global demethylation from around E11.5 to remove their inherited imprinting pattern. During this period, DNA methylation of the somatic cells is maintained (Fig. 1). Demethylation of germ cells is clearly vital if the correct sex-specific epigenetic information is to be subsequently laid down during oocyte and sperm maturation. Demethylation is complete by E13–14, correlating to the period when the male and female mouse PGCs begin to enter mitotic and meiotic arrest respectively. It has been suggested that mitotic/meiotic arrest might necessarily follow demethylation because replication of unmethylated DNA has an increased risk of unrepressed retro-transposons moving and causing mutations (Walsh *et al.* 1998). The time at which this demethylation occurs, and also the amount of methylation lost, appears to be identical regardless of the gender of the embryo (Hajkova *et al.* 2002). Whether the loss of methylation occurs by a passive or active mechanism or a combination of both is not yet known, although the speed with which this occurs would suggest involvement of an active mechanism.

Oocyte development

In female embryos, the gonad forms as an ovary with germ cells forming primordial follicles. As long as the primordial follicle and the oocyte contained within it are not activated to enter the growing population, the methylation level of the oocyte genome remains low and unchanged. It is during the growth phase of the oocyte that the maternal imprints are laid down on the genome (Fig. 1). The imprints are not all established at the same time; instead, each imprinted gene has a specific time at which it will become methylated (Fig. 2). Obata & Kono (2002) analysed parthenogenetic embryos created by nuclear transfer of oocyte nuclei from different stages of follicle development, with the aim of establishing the timing of the maternal imprinting within the oocytes, and showed that *Snrpn*, *Znf127* and *Ndn* genes are imprinted early in follicle development during the primordial to primary follicle stages, whilst imprinting of *Peg3*, *Igf2r* and $p57^{KIP2}$ happens at the secondary follicle stage. There are also genes which become imprinted at even later stages of follicle development, including *Peg1/mest* during tertiary to early antral stages and *Impact* which only becomes imprinted in the oocyte within an antral follicle (Obata & Kono 2002). A further study by Lucifero *et al.* (2004)

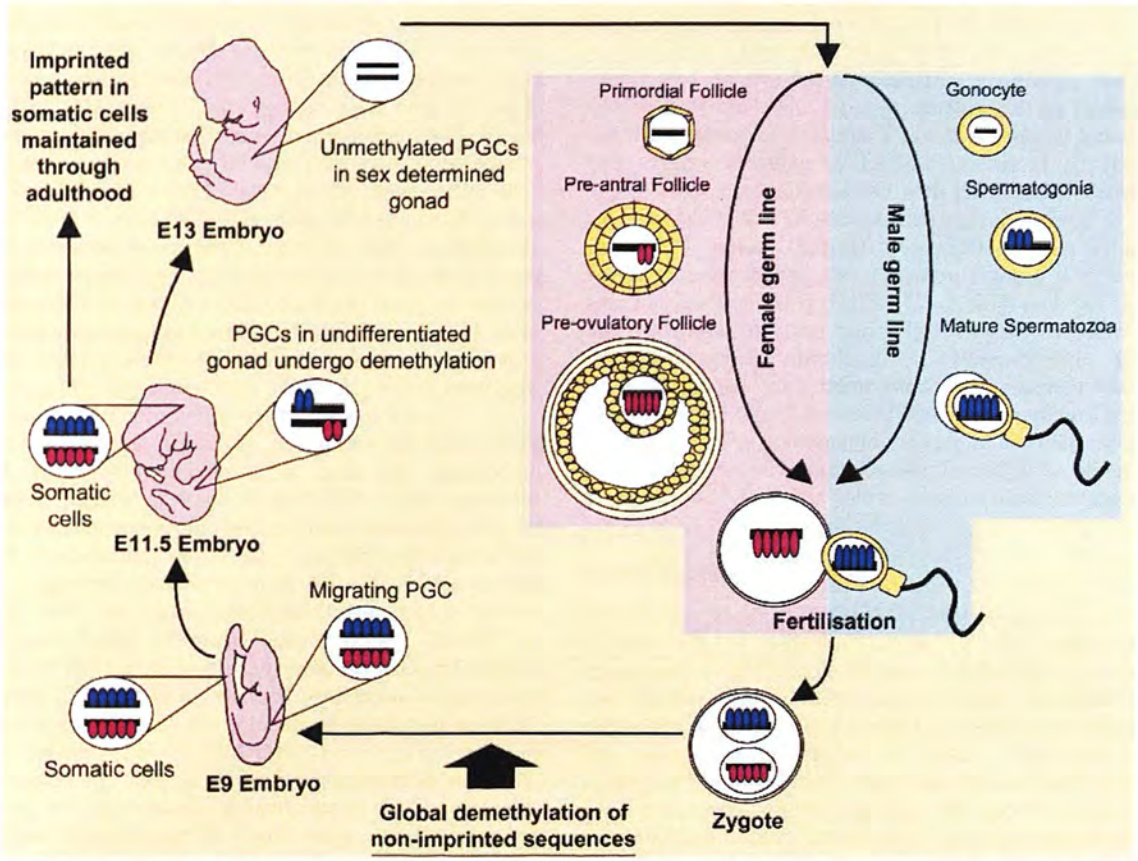

Figure 1 The maternal (pink shaded region) and paternal (blue shaded region) imprints are laid down during germ cell development so that by the time the oocyte and sperm are fully mature the correct pattern of DNA methylation is present on the genome (female imprints, pink ovals; male imprints, blue ovals). After fertilisation (yellow-shaded area), both parental genomes undergo global demethylation of non-imprinted sequences: imprinted genes are protected from this process. During early embryo development the imprinted genes of both the somatic and PGC retain the parental imprints. From E11.5 the primordial germ cells begin to undergo demethylation to erase the inherited parental imprints, but the somatic cells of the embryo maintain the parental imprints through embryo development and into adulthood. The process of PGC demethylation is complete by E13. Subsequent reprogramming of the germ cells occurs when the gender-specific imprinting patterns are once more laid down.

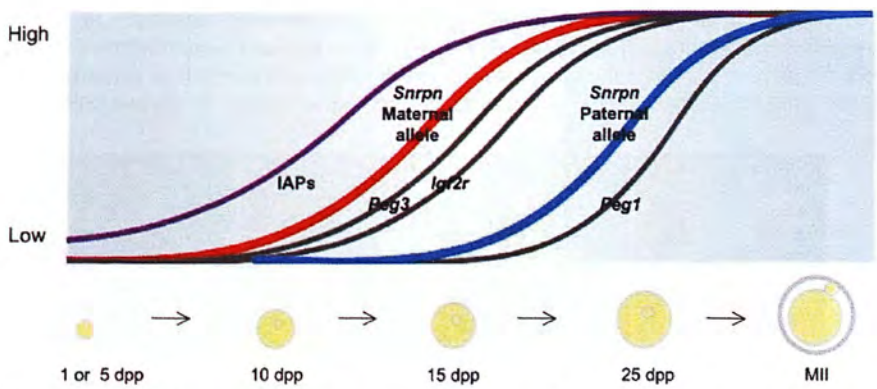

Figure 2 Methylation levels of individual imprinted genes and non-imprinted regions of the genome were assessed over the period of oocyte growth and development. These results demonstrate the gene-specific nature of methylation of the genome, with some genes imprinted early on in follicle development while others are imprinted much later. dpp, days post partum; MII, Metaphase II; IAP, intracisternal A particles; Reproduced from Lucifero *et al.* (2004) with permission from Oxford University Press.

investigated the methylation of imprinted genes by dissecting follicles from ovaries of different postnatal ages and examining the oocytes. This confirmed the earlier Obata & Kono, 2002 study in that *Peg3*, *Igf2r* and *Snrpn* began to gain methylation earlier in development than *Peg1*. By the early antral stages, some differentially methylated regions (DMRs) were fully methylated in all genes other than *Peg1*, while it was not until oocytes were fully mature that *Peg1* appeared to undergo rapid *de novo* methylation. Thus, the imprinting pattern of the oocyte is not fully laid down until it is within a mature follicle ready to ovulate. This has clear implications for assisted reproductive techniques (ARTs), where follicle and oocyte maturation is usually artificially stimulated; any such process must support the correct completion of oocyte imprinting.

Over the period of oocyte growth, the general level of DNA methylation increases as both the appropriate maternal pattern of imprinting is laid down and non-imprinted sequences also become methylated (Fig. 3). The Dnmt involved has yet to be identified, although it has been suggested that one or more members of the Dnmt3 family could be responsible. Dnmt3a, 3b and 3l are all expressed during postnatal oocyte growth. Dnmt3l is expressed at a higher level than either Dnmt3a or Dnmt3b, although all three have maximal expression levels occurring at approximately the same stage of oocyte development (Lucifero *et al.* 2004). Dnmt1s protein is not found in either growing oocytes or in pre-implantation embryos. Instead, an alternatively spliced, more stable transcript, Dnmt1o, is expressed at these stages. In the growing oocyte, Dnmt1o is found in both the cytoplasm and the germinal vesicle (Fig. 4), but once the oocyte is fully matured, it is localised to the cytoplasm where it is stored until it is required during later embryo development (Carlson *et al.* 1992, Mertineit *et al.* 1998). Since *Dnmt1o* translation only occurs early in oocyte development, the stability of this form of Dnmt1 is clearly important.

Dnmt3l does not have the active transmethylase activity which is a characteristic of the other Dnmt3 family proteins, Dnmt3a and 3b. However, Hata *et al.* (2002) found that Dnmt3l expression was vital if normal maternal imprints were to be laid down in the oocyte and that this function may be mediated through its ability to bind and co-localise with both Dnmt3a and 3b. Mice with a disrupted *Dnmt3l* gene are sterile. Males produce no mature sperm (see below); females undergo apparently normal oocyte growth and the resulting oocytes can be fertilised, but the absence of maternal *Dnmt3l* is embryo lethal to heterozygote offspring by E9.5 (Bourc'his *et al.* 2001). Interestingly, a conditional KO with disrupted *Dnmt3a* in the germ cells has an almost identical phenotype to the *Dnmt3l*$^{-/-}$ mouse (Kaneda *et al.* 2004): when females whose oocytes lacked *Dnmt3a* were crossed with wild-type males all offspring died by E11.5, with embryos lacking methylation on the normally methylated maternally imprinted genes (resulting in inappropriate gene expression). This study demonstrates the essential role of *Dnmt3a* in the establishment of maternal imprints. Kaneda *et al.* (2004) also investigated the role of *Dnmt3b* using a conditional KO; these animals were found to be phenotypically normal and were able to produce viable offspring.

Sperm development

As with the oocyte, new imprints are laid down as sperm develop (Fig. 1), with the increase in DNA methylation levels not just attributable to the establishment of paternal imprints but also the methylation of other non-imprinted sequences, such as intracisternal A particles (IAPs) becoming methylated (Walsh *et al.* 1998). The paternally expressed (i.e. maternally imprinted) human *MEST/PEG1* gene is demethylated during fetal life and then remains unmethylated through all stages of sperm development in adult life. Ueda *et al.* (2000) analysed the methylation level of an imprinted gene, *H19*, in male germ cells and found that the *H19* imprint is laid down early in germ cell development before meiosis occurs. The same result was found in humans, with the *H19* gene becoming methylated before meiosis at the spermatogonial stage of development (Kerjean *et al.* 2000). In general, though, there is less information about the laying down of imprinting patterns during sperm development compared with what is known about imprinting in oocytes.

The resumption of mitotic division of male germ cells at puberty coincides with an increase in the level of Dnmt1 within the spermatocytes. During the early stages of

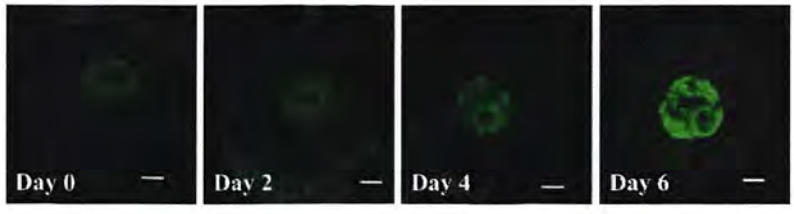

Figure 3 Global methylation level of the oocyte increases over the period of growth and development associated with follicle development from pre-antral stages to full maturity. Confocal images showing oocytes stained with 5-methyl-cytosine antibody (Eurogentec, Seraing, Belgium) and an FITC fluorescent secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Late pre-antral follicles were dissected from 3-week-old mouse ovaries and cultured as in Spears *et al.* (1994), with follicles developing to the Graafian stage over a 6-day period. Follicles were removed from culture and oocytes recovered on days 0, 2, 4 and 6 of culture before being fixed and stained. White scale bars represent 10 μm.

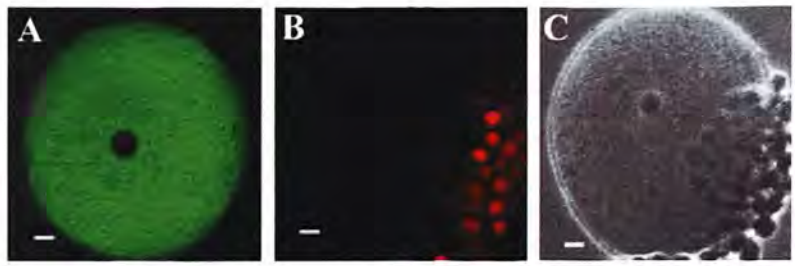

Figure 4 The oocyte expresses only Dnmt1o (and not Dnmt1s). Until oocyte maturation is complete, Dnmt1o is localised in the cytoplasm and germinal vesicle – but not the nucleolus – of growing oocytes. The figure shows an oocyte from a mid-antral (i.e. not yet fully mature) follicle. (A) Immunocytochemistry using the PATH52 antibody that recognises both Dnmt1s and Dnmt1o (kindly donated by T Bestor, Columbia University). The image shows localisation of Dnmt1 in both the cytoplasm and the germinal vesicle of the oocyte, with only the nucleolus remaining unstained. (B) Immunocytochemistry using the UPT82 antibody which detects only Dnmt1s (kindly donated by J R Chaillet, University of Pittsburgh). The cumulus cells are heavily stained while, as expected, the oocyte remains unstained showing that staining in (A) was specifically due to Dnmt1o. (C) Transmitted light image of oocyte shown in (B). White scale bars represent 10 μm.

meiosis the level of Dnmt1s in spermatocytes is high but a reduction in the level of the Dnmt1 enzyme has been observed in pachytene stage spermatocytes (Jue *et al.* 1995). This is due to the expression of an alternatively spliced version, *Dnmt1p*, which does not appear to be translated. Although Dnmt1s within the sperm is normally found in both the nucleus and the cytoplasm, it is concentrated at nuclear foci during some stages of meiosis and it may be that this correlates with the laying down of paternal imprints (Jue *et al.* 1995). Dnmt3l is expressed in the murine testes from E12.5 in non-dividing prospermatogonia with peak expression seen at the time of birth, after which there is a dramatic postnatal reduction in expression level (Bourc'his & Bestor 2004, La Salle *et al.* 2004). Dnmt3a expression in the testis is raised before birth and during early postnatal life, in contrast to the level of Dnmt3b expression which is lower during embryonic life and rises postnatally (La Salle *et al.* 2004). Mice lacking *Dnmt3l* have smaller testes, and by adulthood there are virtually no spermatozoa present, resulting in sterile animals (Hata *et al.* 2002). *Dnmt3l* is required if normal meiosis and silencing of retrotransposons is to occur (Bourc'his & Bestor 2004). The loss of *Dnmt3a* results in a similar although less extreme phenotype than that seen in the *Dnmt3l* KO mouse (Hata *et al.* 2002). More recently, the male *Dnmt3a* conditional KO was created, with no germ cell Dnmt3a expression but with somatic cell levels maintained (Kaneda *et al.* 2004). Spermatogenesis is severely impaired in these mice so that by 11 weeks of age there are no spermatozoa in the testis, demonstrating a vital role for *Dnmt3a* in this process. Offspring from these conditional KO males have errors in the methylation of some paternally imprinted genes.

Fertilisation and early embryo development

As shown in Fig. 1, the fertilised embryo contains methylated DNA, some of which will be located in imprinted genes (both maternal and paternal) while the majority of the DNA methylation will be positioned on non-imprinted sequences (again of both maternal and paternal origin). Early on in embryo development, the embryo loses its methylation at the non-imprinted DNA sequences; it now appears that this DNA methylation is lost in a parent-of-origin specific order, at least in some species. Imprinted genes are resistant to these early demethylation processes. The embryo's germ cells will lose methylation of the imprinted genes during gonadal development, while somatic cells maintain these methylation patterns throughout embryonic development and, in the main, throughout the life of the newly formed organism (although imprinted patterns are lost or altered in some tissues, such as the liver; McLaren & Montgomery 1999).

In some species (such as the mouse), the paternal genome is actively demethylated immediately after fertilisation (Oswald *et al.* 2000). The occurrence and degree of this demethylation appears to be species specific, and its regulation is currently unknown. Cross-fertilisation using gametes from several species of animal has shown that, although there are sperm characteristics which affect the degree of demethylation, the main factor determining whether the paternal genome becomes demethylated is oocyte specific (Beaujean *et al.* 2004). In the search for the factor responsible for this post-fertilisation active demethylation, MBD2 was proposed as a candidate after an *in vitro* study by Bhattacharya *et al.* (1999). However, a subsequent study utilising *MBD2* $^{-/-}$ oocytes has found that the rapid demethylation of the paternal genome still occurs in its absence (although this result does not eliminate the possibility of redundancy; Santos *et al.* 2002). The maternal genome undergoes passive demethylation which is slower to occur and is linked to the replication of DNA in the absence of any maintenance methylase activity. Although the general trend after fertilisation is for non-imprinted sequences to undergo demethylation, there does appear to be some specific incidences of *de novo* methylation such as the DMRs of the *Dnmt1o* gene in the one-cell to blastocyst stage embryo (Ko *et al.* 2005).

Dnmt1o remains localised to the cytoplasm of the embryonic cells at all pre-implantation stages with the exception of the eight-cell embryo. During this stage, the protein has been shown to translocate to the nuclei, where it is thought to play a role in maintaining the methylation level of imprinted genes. Dnmt3a is expressed by pre-implantation embryos and there is no evidence of the protein being excluded from the nucleus at any developmental stage (Ko *et al.* 2005). Although *Dnmt3b* is not transcribed in the pre-implantation embryo the protein is present at all stages from the one-cell to the blastocyst stage; while mainly localised to the cytoplasm, it is not fully excluded from the nucleus (Ko *et al.* 2005).

Until recently, embryos which consisted of two maternal or two paternal genomes were unable to develop to term. Work examining the competence of parthenogenetic embryos found they were able to develop at best until E9.5. By creating an embryo with one set of chromosomes from a fully grown and the other from a non-growing oocyte, Kono *et al.* (1996) showed that embryo development could be extended to E13.5. It was thought that this increase in the length of time the embryo survived was due to the ability of the non-growing oocyte chromosomes (with, therefore, no female imprints yet laid down) to partially compensate for the lack of a paternally imprinted set of chromosomes. Parthenotes which died at E9.5 and E13.5 had incorrect biallelic expression of the normally monoallelic *H19* gene. The most recent work by Kono *et al.* (2004) has resulted in the birth and survival of a viable parthenote. This was achieved using non-growing oocytes from a transgenic mouse with a 13 kb deletion of *H19*. The mouse had H19 expression consistent with that normally seen from the paternal genome. As *H19* in turn influences the expression of the imprinted *Igf2* gene, the embryo had monoallelic expression of both *H19* and *Igf2* (from the wild-type fully grown oocytes only), as would be the case in normal embryos. The fact that normal embryo development is possible after the female genome is altered to more closely resemble the genomic imprinting pattern of the male genome demonstrates the importance of this mechanism in controlling development, reinforcing the idea that the presence of mammalian genomic imprinting might act as a barrier to parthenogenesis (see above).

Errors in genomic imprinting

With genomic imprinting being a basic mechanism clearly vital for many aspects of development, there are, not surprisingly, many instances of developmental defects due to imprinting errors whether occurring naturally or during human intervention (Table 1).

Assisted reproductive techniques

In recent years, there has been increasing concern that children conceived with the aid of ARTs could have an increased occurrence of disorders linked to imprinting problems. At the turn of the century, two studies (Cox *et al.* 2002, Orstavik *et al.* 2003) reported the occurrence of three children conceived using intra-cytoplasmic sperm injection (ICSI) with Angelman syndrome (AS), a neurological disorder characterised by developmental delay and seizures, suggesting that the risk of AS may be increased by the use of ICSI. The fear of such a link was then increased with three studies that examined patients with Beckwith–Wiedemann syndrome (BWS) to see if a higher than expected proportion of these cases came from ART babies; all studies found a disproportionate number of such cases (DeBaun *et al.* 2003, Gicquel *et al.* 2003, Maher *et al.* 2003). BWS is characterised by both pre- and postnatal overgrowth and defects of the abdominal wall. Children who had been conceived using ART and suffered from BWS had the methylation status of their *H19* and *LIT1* genes established, with only one of the identified children demonstrating normal methylation patterns on both these genes. Data suggest that ART results in a three- to sixfold increase in the incidence of the normally rare BWS, although some of the studies may in fact be underestimating the true risk (DeBaun *et al.* 2003).

The cause of the link between ARTs and imprinting disorders is currently unknown. It could be due to some aspect of the ARTs involved. There is a wide range of different ARTs which are now routinely used within clinics. Techniques might expose one or both of the germ cells to an altered hormonal regime *in vivo*, a period of time in culture or mechanical manipulation. Any such alterations to the normal environment of the oocyte or sperm could result in changes to some aspect of their imprinting mechanisms. Alternatively, it could be due to some error within the germ cells used, bearing in mind that couples seeking to use ARTs have reduced natural fertility.

The potential problems do not end with the germ cells; the pre-implantation embryo is also often exposed to a period of culture which could again alter the epigenetic reprogramming known to occur at these early stages. One such example is abnormal biallelic *H19* expression of mouse embryos cultured in Whitten's media (Doherty *et al.* 2000). It is not just in humans and mice that potential problems with imprinting have been seen. In large domestic mammals such as sheep and cattle, large offspring syndrome (LOS) was identified when embryos had been exposed to some time in culture (Young *et al.* 1998). Further investigations into LOS in sheep have identified changes in the expression level of the imprinted gene, *IGF2R*, due to epigenetic changes (Young *et al.* 2001). Similar overgrowth problems seen in mice and humans are often caused by errors in several imprinted genes including *Igf2* and *H19* (Eggenschwiler *et al.* 1997), suggesting that other genes responsible for fetal growth and development could be involved in LOS.

If sperm used for *in vitro* fertilisation have lowered global methylation levels there is no alteration in either fertilisation rate or in early embryo quality; however, there is a reduction in pregnancy rate, demonstrating the importance

Table 1 Diseases and syndromes which result from problems to the imprinting mechanisms or from errors in the imprinting of genes.

Disorder	Affected genes	Phenotype	Art link?
Angelman syndrome	Chromosome 15 – maternal copy, loss of *SNRPN* imprinting	Mental retardation, ataxic gait, seizures, sociable disposition	Yes
Autism	Unknown X-linked gene (not always connected to imprinting)	Impaired language development, problems with social and motor skills	
Beckwith–Wiedemann syndrome	11p15 region – altered expression of *IGF2*, *H19* and *LIT1*	Undescended testes, large newborn, seizures, abdominal wall defects	Yes
Cancer	Variable, e.g. *IGF2* in lung cancer (not always connected to imprinting	Tumours	
ICF (immunodeficiency, centromeric region instability and facial anomalies syndrome)	*DNMT3B*	Immune problems, facial anomalies, growth retardation	
Paraganglioma	Paternal mutations *SDHA* (*PGL1*) and *PGL2*	Glomus tumours of the parasympathetic ganglia mainly in the head and neck region, tend to be slow growing and benign	
Prader–Willi Syndrome	Chromosome 15 – paternal copy	Undescended tests, mental retardation, short stature, obesity small hands and feet	
Pre-eclampsia	Not yet defined	Serious complication of pregnancy	
Pseudohypoparathyroidism type IA (Albright hereditary osteodystrophy)	Imprinted *GNAS* cluster	Parathyroid horomone resistance, short stature, round face and short hand bones	
Pseudohypoparathyroidism type IB	Imprinted *GNAS* cluster	Parathyroid hormone resistance localized to renal system, causing hypocalcaemia and hyperphosphataemia	
Rett syndrome	MeCP2	Childhood neurodevelopmental disorder mainly affecting females. Loss of motor function and mental retardation	
Silver–Russell syndrome	Cases which are imprinting related – chromosome 7	Short stature, excessive sweating, triangular face, inward curving 5th fingers and coloured spots on the skin	
Transient neonatal diabetes	An imprinted gene at 6q24. Candidates are *ZAC* & *HYMAI*	Growth retardation and diabetes which develops during the first 6 months of life but corrected by 18 months	
Turner syndrome	Complete or partial loss of second X chromosome	Affects females – short stature, social problems and ovarian failure	
Wilms' tumour	*IGF2* loses imprinting	Childhood kidney tumour	

of normal gamete DNA methylation on embryo development and ultimately ART outcome (Benchaib *et al.* 2005). There is recent evidence that sperm obtained from males with low sperm counts due to abnormal spermatogenesis have incorrect genomic imprinting (Marques *et al.* 2004), although such sperm can then be used, for example, in ICSI. Marques *et al.* (2004) found that, although the maternal imprints had been erased from all sperm, the paternally methylated *H19* gene was under-methylated in some sperm from the oligozoospermia donors. Any embryo derived from one of these hypomethylated sperm could have inappropriate expression of the imprinted *H19* and *IGF2* genes, the effect of which is not known.

Cloning

Studies investigating the failure of cloned animals have also turned their attention to the role of genomic imprinting. The fact that many of the errors seen in cloned animals have epigenetic causes has been demonstrated by examining the offspring of cloned mice. These cloned mice were obese but this trait was not passed onto the offspring, demonstrating that this was not a genetic error but due to epigenetics. This finding is important as it suggests that, despite any problem in the cloned animals, it is possible that their germ cells are able to correctly undergo genomic imprint reprogramming (Tamashiro *et al.* 2002). In bovine cloned embryos, it has been found that the levels of methylation in the cells of the embryo are higher than normal at the four-cell and eight-cell stages. Although there is initial demethylation of the donor genome, passive demethylation does not occur to the level seen in normal embryos. In addition to a reduction in the amount of demethylation, there also appears to be inappropriate *de novo* methylation occurring at early stages of embryo development (Dean *et al.* 2001). It is also possible that errors in the Dnmt enzymes normally present in the early embryo could account for alterations in methylation seen in these embryos. Analysis of cloned mouse embryos shows inappropriate presence of Dnmt1s within the pre-implantation embryo; this transcript of Dnmt1 is never present in normal embryos. It was also observed that at the eight-cell stage, when Dnmt1o would normally translocate into the nuclei of embryonic cells, some nuclei within each embryo were devoid of any Dnmt1 transcript, suggesting that these cells are unable to maintain normal methylation patterns (Chung *et al.* 2003).

Disease

In some cases, imprinting errors can occur which, although not embryo lethal, cause abnormal physiological processes and lead to disease. Such diseases can arise when any imprinted gene becomes hypermethylated or hypomethylated. Effects are not always limited to the loss of function of a single gene, as some imprinted genes affect the expression of other genes, such as *H19* and *IGF2*. The linked Prader–Willi syndrome (PWS) and AS are examples of disorders that can occur when correct imprinting is lost. A loss of a currently unidentified imprinted gene results in PWS when the deletion is paternally inherited, whereas the same errors cause AS to develop when maternally transmitted (Moncla *et al.* 1999). Other examples of diseases which result after incorrect imprinting include BWS, Silver–Russell syndrome and transient neonatal diabetes.

Disease can also result from defects in mechanisms regulating imprints. One of the key groups of enzymes with a role in genomic imprinting are the Dnmts which are responsible for the addition of methyl groups to the DNA. When problems arise within this aspect of the imprinting mechanism it can lead to disease in the individual. One such example is immunodeficiency, centromeric region instability and facial anomalies syndrome which is a result of a mutation in *DNMT3B* (for review see Ehrlich 2003).

Another major component of the imprinting mechanism is the family of methyl-binding domain proteins. MeCP2 is a protein which contains a methyl-binding domain. It has a role in controlling the transcription of imprinted genes through its ability to bind to methylated DNA. The importance of this protein for normal development and physiological function is demonstrated by Rett syndrome which occurs when *MECP2* is mutated (Amir *et al.* 1999).

There are some diseases with multiple causes which only in some cases involve errors to the imprinting mechanism or alterations to imprinted genes. Cancer is one such disease, with some cases of cancer being identified as having a cause linked to genomic imprinting while many other incidences of the disease occur because of unrelated problems. In some instances, human tumour cells have been found to overexpress one or more of Dnmt1s, 3a and 3b, with the largest upregulation occurring to Dnmt3b (Robertson *et al.* 1999). These results support the previous observations of abnormal methylation levels seen in tumour cells. One cancer which demonstrates such raised Dnmt levels is acute myelogenous leukaemia; it may be that this overexpression of the Dnmt enzymes accounts for the hypermethylation and silencing of an important tumour suppressor gene (Mizuno *et al.* 2001).

Conclusion

Genomic imprinting is a gene transcription control mechanism which is vital for normal healthy offspring. Although in recent years there has been a huge volume of work undertaken to elucidate the mechanisms behind genomic imprinting there are still many unanswered questions. Recent data have demonstrated that there are species differences in the imprinting mechanism which still need to be fully explored but could have implications for the success of cloning attempts. Additionally, knowledge of genomic imprinting may aid the understanding of some human diseases and offer potential therapies. The field of ART will also benefit from a greater understanding of genomic imprinting, resulting in improved techniques with an increased success rate and, most importantly, a safer outcome.

Acknowledgements

Support by MRC and BBSRC is gratefully acknowledged, the authors declare that there is no conflict of interest that would prejudice impartiality of this scientific work

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Received 26 July 2004
First decision 17 September 2004
Revised manuscript received 26 Jan 2005
Accepted 26 May 2005

Follicular growth and oocyte competence in the in vitro cultured mouse follicle: effects of gonadotrophins and steroids

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Running Title - Steroids and Oocyte Competence

Keywords – DNA methylation/gonadotrophins/oocyte competence/steroids

Abstract

Although there have been extensive studies on the effects of gonadotrophins and steroids on follicular development, less is known as to the effects these hormones have on the acquisition of oocyte developmental competence. This study investigates the effect of altering the gonadotrophin or steroidal environment on follicular development and on oocyte viability and DNA methylation. Oocytes were obtained from pre-ovulatory follicles after individual follicle culture from the pre-antral stage; gonadotrophin or steroid levels were manipulated during the culture period. Oocytes obtained from follicles grown in gonadotrophin free conditions were able to fertilize and develop to the blastocyst stage despite their impaired follicle development. There was no effect of LH or steroids on follicular growth. Altering the steroidal environment did however, affect oocyte development. Raising androgen and oestrogen levels lowered fertilisation rates while increasing the level of global DNA methylation. This study demonstrates that gonadotrophins and steroids influence the acquisition of developmental competence of the oocyte and suggests that optimal steroid exposure during follicle development is required for the oocyte to mature correctly.

Introduction

The roles that follicle stimulating hormone (FSH) and luteinizing hormone (LH) play in ovarian development have been extensively studied for a number of years. Until the recent availability of recombinant forms of both gonadotrophins it was difficult to define the precise and individual roles that FSH and LH played in follicular growth and development. From the growing number of reports that have now utilized these recombinant gonadotrophins it is clear that FSH on its own is capable of promoting follicular growth and development, while LH augments steroidogenesis and plays a critical role in the ovulatory process (Chappel and Howles 1991, Hillier 1994). Recombinant FSH alone has been used in both clinical and animal studies: results have indicated that inclusion of LH in ovarian stimulation protocols is required for adequate steroidogenesis and that inadequate oestrogen synthesis is detrimental to embryo viability and endometrial development (Balasch *et al.*, 1995, Spears et *al.*, 1998, Balasch *et al.*, 2001 Shoham, 2002). The concentration of LH required to optimize stimulation protocols has been the subject of many debates. It has been suggested that follicular maturation is optimal when LH levels do not rise above a 'ceiling' which, when exceeded, leads to an arrest of follicular development (Loumaye *et al.*, 2003). Elevation of LH levels above basal rates during ovarian stimulation protocols has also been proposed to impact directly on oocyte quality (Balasch *et al.*, 2001, Shoham, 2002). Other studies, however, have failed to confirm any correlation between elevated LH levels and pregnancy rates (Westergaard *et al.*, 1996). From these conflicting reports it is still unclear what effect LH has on oocyte development within the follicle prior to ovulation and how this affects the quality of the resulting embryo after fertilisation.

Gonadotrophin levels will directly affect ovarian steroidogenesis, LH stimulating the thecal cells of the follicle to produce androgens which are subsequently aromatized to oestrogens within the FSH-responsive granulosa cells. Androgens and oestrogens have been proposed as paracrine/autocrine regulators of follicular development, but, as with LH, there is controversy over their roles in the ovary. Apart from serving as substrates for oestrogen synthesis, androgens have been implicated in promoting follicular development (Murray *et al.,* 1998, Vendola *et al.,* 1998), and in up-regulating FSH receptor expression and enhancing FSH-stimulated follicular differentiation (Tetsuka and Hillier, 1997, Weil *et al.,* 1999). However, excess androgen levels have also been associated with inducing follicular atresia, while high androgen:oestrogen ratios in follicular fluid have been correlated with poor fertilization and development rates (Andreisz and Trounson, 1995, Xia and Younglai, 2000). Oestrogens have been shown to inhibit follicular atresia and to facilitate the proliferation of granulosa cells, the actions of gonadotrophins, gap junction formation and steroidogenesis (Merck *et al.,* 1972, Roberts and Skinner, 1990, Billig *et al.,* 1993, Dorrington *et al.,* 1993, Bley *et al.,* 1997). Other studies have indicated that oestrogen is not obligatory for follicular development (Spears *et al.,* 1998, Balasch *et al.,* 1995). As both androgen and oestrogen receptors have been reported on the oocyte, it is possible that steroids may also exert a direct effect (Szlotys *et al.,* 2003, Gill *et al.,* 2004).

Recently the phenomenon of genomic imprinting has received much attention, with maternal epigenetic imprints established during the oocyte growth period (Obata *et al.,* 1998). Alterations to the mechanisms involved in imprinting can result in early embryonic lethality, but may become evident only during the later life of the

organism. One of the most researched aspects of genomic imprinting is the role of DNA methylation in controlling gene transcription, with imprinted genes in the paternal and maternal genomes differentially methylated (Simon *et al.,* 1999, Lucifiro *et al.,* 2002). The signals that mark certain genes for imprinting either via methylation or by other means has yet to be elucidated, however paracrine signals arising from gonadotrophin or steroidal actions within the follicle are possible candidates.

It has been proposed that the oocyte needs to be exposed to the correct sequence and pattern of steroids in order to acquire the molecular programming required for fertilisation and further development (Moor *et al.,* 1998). Alterations to the normal follicular sequence of FSH- and LH-stimulation, such as occurs during ovulation stimulation, will inevitably perturb the pattern of steroid exposure which may in turn have an effect on oocyte competence. This study aims to investigate the effects of varying gonadotrophin and steroidal treatments on follicular development, oocyte viability and DNA methylation, using an in vitro system that permits the development of individual follicles from the pre-antral to the Graafian stage. This system is highly physiological in that it closely mimics follicular development in vivo. While mechanical isolation of follicles is labour-intensive and imposes some restrictions, it does ensure that individual follicles, at a similar developmental stage can be placed in defined conditions thereby negating the effects of any extra follicular factors.

Materials and Methods

Animals

F1 (C57BL/6J x CBA background) and *hypogonadal* (*hpg*) mutant mice were housed in an environmentally controlled room on a 12h light: 12h dark photoperiod. Food and water were available *ad libitum*. *Hpg* mutant animals were generated by breeding heterozygous pairs ($hpg^{+/-}$). Offspring were genotyped by extracting DNA from an ear clipping which was then analyzed by polymerase chain reaction (Lang, 1991). All animals were maintained and treated according to UK Home Office requirements and the study received local ethics committee approval.

Culture Supplements

In order to accommodate for batch to batch variation in recombinant human follicle stimulating hormone (rhFSH) potency, batches were titrated to ensure that each batch was used at the minimum concentration that allowed follicles to reach sizes of >400 μm over a 5-6 day period (doses are described for individual experiments).

The use of serum in the culture media could potentially introduce additional gonadotrophins and/or steroids that could influence the results. In earlier experiments, serum was obtained from *hpg* mutant adult mice (which have no or very low levels of circulating gonadotrophins). Briefly, blood was withdrawn from anaesthetized adult animals via cardiac puncture, allowed to clot for a minimum of 10 minutes then centrifuged at 13K rpm for 10 minutes. Serum from each batch of animals was pooled before being aliquoted and stored at -70°C. As *hpg* serum proved erratic in its ability to support fertilization, charcoal-stripped fetal bovine serum

(CSS) was used in later experiments. The use of activated charcoal to remove steroids has been previously described (Huot and Shain, 1988). Due to the slower maturation of follicles in this serum, the culture period prior to oocyte collection for in vitro fertilisation (IVF) was extended to 5 days.

Culture Medium and Chemicals

All chemicals were supplied by the Sigma Chemical Company (St Louis, USA) with the exception of those noted here. Epidermal Growth Factor (EGF) was from Roche (Lewes, UK) and silicone fluid was from Merck (Lutterworth, UK). Arimidex was a gift from Astra Zeneca (Macclesfield, UK). Recombinant gonadotrophins (rhFSH and rhLH) were supplied by Serono (Geneva, Switzerland). Liebovitz L-15 and α-minimal essential medium (α-MEM) were supplied by Invitrogen (Renfrew, UK). Fetal bovine serum was supplied by Labtech (Uckfield, UK). 5-methyl cytosine antibody was supplied by Eurogentec (Seraing, Belgium). TRIT-C conjugated secondary antibody was supplied by Jackson ImmunoResearch (Pennsylvania, USA). Vectashield was supplied by Vector Labs. (Peterborough, UK).

Standard Follicle Culture

21-25 day old F1 female animals were sacrificed by cervical dislocation, their ovaries removed and placed in watch glasses containing Leibovitz L-15 medium supplemented with 3mg/ml^{-1} BSA (dissection medium). Individual pre-antral follicles (175 ± 15 μm) were dissected and cultured as previously described (Murray *et al.*, 1998). On average 25-30 follicles were obtained from each mouse and these

were randomly allocated across all treatment groups. Follicles were incubated for periods of between 4-6 days in a humidified 5% CO_2, 95% air incubator at 37°C. Media were supplemented with 5% serum and gonadotrophins or steroids as detailed for each experiment.

Follicular growth, morphology and steroid production

Follicles were examined daily; antral formation was noted and follicles were measured using an ocular micrometer. At the end of the culture period, spent culture media were frozen at –20°C until analyzed for steroids. Samples of fresh medium containing all supplements were also frozen and analyzed for steroids. The various media from the follicle cultures were then analyzed for the presence of oestradiol and androstenedione. Oestradiol was measured by ELISA as previously described (Murray *et al.*, 1998). Androstenedione was measured by radioimmunoassay as previously described (Hillier *et al.*, 1991).

Oocyte Maturation and In Vitro Fertilisation

Oocytes from uncultured pre-antral follicles or cumulus-oocyte complexes (COCs) from cultured follicles were dissected and transferred to α-MEM medium supplemented with 20ng ml^{-1} EGF, 5% serum and rhFSH. The serum type was the same as that used in the follicle culture. The COCs were then incubated for 18hr before being transferred into droplets of T6 medium to await fertilisation (Quinn *et al.*, 1982). Spermatozoa suspensions were prepared from F1 males as described previously and allowed to capacitate for 2hr (Spears *et al.*, 1994). Spermatozoa were added to the droplets of T6 medium containing the oocytes. After 4-5 hours

incubation the oocytes were transferred into droplets of glutamine-free KSOM medium supplemented with $1mg/ml^{-1}$ fatty acid free BSA (Devreker and Hardy, 1997). As a control for the IVF system, oocytes were obtained from 6-week old F1 females that had been superovulated as described previously (Spears *et al.*, 1994): oocyte-cumulus complexes were recovered from the oviducts, transferred into droplets of T6 medium and treated in the same manner as experimental oocytes. All oocytes were examined the following day and the number of 2-cell embryos counted. These were transferred to fresh drops of KSOM medium at a ratio of 1 embryo to 2µl of medium. A maximum of ten x 2-cell embryos were transferred to a 20µl drop of medium. Where appropriate more than one 20µl droplet of medium was set up and if necessary the droplet volume was adjusted for the number of 2-cell embryos found. All droplets were covered with silicone fluid and incubations were in an environment of 5% CO_2 in air, at 37°C. These were then incubated until the blastocyst stage of development. The number of oocytes fertilising and reaching the blastocyst stage of development was noted.

Methylation Immunohistochemistry

At the end of the culture period, COCs were isolated by rupturing the follicles with fine needles. Oocytes were then denuded by repeated pipetting. Oocytes were fixed overnight in paraformaldehyde (4% in PBS) at 4°C before being permeabilised in Triton-X100 (0.5% in PBS) for 30 minutes and then denatured with 4N HCl at 37°C for 1 hour. Non-specific binding was blocked using BSA (2% in PBS) for 1 hour. Oocytes were then incubated with a primary 5-methyl cytosine antibody (1:50 in PBS/BSA) for 1 hour at 37°C, washed and stained with a FITC-C conjugated

secondary antibody (1:200 in PBS/BSA) for 1 hour. After a final wash (Tween20, 0.05% in PBS), the oocytes were mounted on positively charged slides using vectashield.

Confocal Microscopy

At the end of experiments examining follicular growth, representative follicles from each of the groups were labeled to detect apoptotic cells using the Terminal Transferase-mediated dUTP Nick End labeling (TUNEL) technique and counterstained using propidium iodide (Baker *et al.,* 2001). Follicles were examined using the Leica TCSNT Confocal microscope (Leica Microsystems, Milton Keynes, UK) using a 63X water-corrected PL APO lens. Simultaneous scans at 488 and 568nm were taken to produce an amalgamated, true color RGB image.

Oocytes from experiments to examine methylation were serially scanned at 3μm intervals through the stained region, using a 40X lens. The average threshold of the images was calculated using Imaris imaging software. Using this information, global DNA methylation levels were assessed with the Scion Image Software package. Specificity of staining was checked by examination of oocytes exposed only to secondary antibody. To allow comparisons between experimental runs, all raw data were normalized by comparison to the mean of the control group from that run.

Statistics

Hormone assays were analyzed by ANOVA followed by Tukey-Kramer multiple comparison tests where appropriate. IVF results were analyzed by chi-square. Methylation levels were analyzed by ANOVA, followed by unpaired t-tests where appropriate.

Results

Experiment 1; The effects of altered gonadotrophin regimes on follicular growth, development and subsequent oocyte developmental competence

Experiment 1A: Follicular Growth and Development.

Follicles were allocated to control medium supplemented with 5% *hpg* serum either without gonadotrophin supplements (**Gn free**) or into the following treatment groups: **FSH alone** (control medium + 5 IU ml^{-1} rhFSH); **FSH + low LH** (control medium + 5 IU rhFSH + 0.01 IU rhLH ml^{-1}); **FSH + high LH** (control medium + 5 IU rhFSH + 0.05 IU rhLH ml^{-1}). Follicles were moved into fresh wells daily and cultured for 6 days. The experiment was performed twice, with 30-40 follicles cultured per treatment. Damaged follicles were discarded within the first two days of the culture period.

FSH alone was capable of inducing follicular growth and development: the addition of low or high LH had no effect on the development of the follicles, with follicles reaching the same size and antral formation occurring at the same time and to the same extent in all gonadotrophin groups (Fig. 1). Gn free follicles initially began to grow, although growth was significantly restricted by day three of culture when compared to all other group ($p<0.001$). These follicles then began to regress from day 4 onwards and no follicles with antral cavities were seen in this treatment (Fig. 1).

Representative follicles from all groups were labeled for apoptosis using TUNEL labeling and examined by confocal microscopy (Fig. 2). The only group of

follicles that exhibited any high degree of apoptotic labeling were those in the Gn free group, with most granulosa cells near the basement membrane showing TUNEL labeling (Fig. 2A). In follicles from all other groups, any TUNEL labeled cells were almost always situated around the antral cavity; this is likely to indicate a healthy follicle: as the antral cavity enlarges, granulosa cells nearest to the cavity become apoptotic and release their contents into the antral fluid (Baker *et al.,* 2001).

Spent culture media from days 4 and 5 of culture were analyzed for the presence of oestradiol and androstenedione. Gonadotrophin treatment altered the production of androstenedione and oestradiol during follicular growth (Fig. 3). Neither steroid was detectable in unincubated culture medium or in that from the Gn free group (data not shown). Follicles from both the FSH + low LH and the FSH + high LH groups were producing androstenedione on day 4 of culture, whereas no steroid was detected in the medium until day 5 of culture when follicles were treated with FSH alone ($p<0.05$, Fig. 3A). This was reflected in the oestradiol results, as both the FSH + low LH and the FSH + high LH groups produced significantly higher concentrations of oestradiol compared to the FSH alone group ($p<0.05$, Fig. 3B). While androstenedione production increased significantly in the FSH alone, FSH + low LH and the FSH + high LH groups by day 5, there was a marked increase in the production of this steroid by follicles cultured with FSH + low LH when compared to either the FSH alone or the FSH + high LH group (Fig. 3A). Similar levels of oestradiol were produced at this time point irrespective of treatment (Fig. 3B).

Experiment 1B: In Vitro Fertilisation.

Follicles were allocated to treatment groups as described above in Experiment 1A (i.e. Gn free, FSH alone, FSH + low LH and FSH + high LH). These were cultured for 4 days, after which the COCs were placed in maturation medium prior to IVF. Maturation medium was supplemented with 1 IU ml^{-1} rhFSH. Four experimental runs were performed with a total of 160 follicles cultured in each treatment. The number of oocytes available for IVF at the end of the culture period was as follows: Gn free -114; FSH alone - 123; FSH + low LH - 128 and FSH + high LH - 98. A further group of 30 follicles were dissected between 200-300μm (the same size as that attained by the Gn free group at the end of the culture period). These follicles were not cultured: instead the oocytes from these follicles were removed immediately and placed into maturation medium before undergoing IVF. There was little difference in fertilisation rate amongst the culture groups regardless of treatment (Fig. 4A). In contrast, later embryo development was more variable. The percentage of two-cell embryos developing to the blastocyst stage was similar in the FSH alone and FSH + low LH group but there was a substantially lower rate of two-cell embryos derived from the FSH + high LH group that developed into blastocysts (Fig. 4B). This low percentage was similar to the percentage of blastocysts derived from the Gn free group. The differences between all groups did not, however, reach significance ($P = 0.08$). In comparison to the 20% fertilisation rate and 23.5% blastocyst rate obtained from the Gn free group oocytes, 29 out of 30 of the oocytes from the uncultured late preantral follicles did not fertilise, while the remaining oocyte did not proceed past the 2-cell stage even though both groups of oocytes were obtained from similar sized follicles. It appears, therefore, that the oocytes from the Gn free group had undergone a degree of maturation during the culture period

despite the fact that they were from atretic looking follicles. Results from superovulated F1 mice (198 oocytes) are given for comparison.

Experiment 2: Effect of alteration in steroid regime during follicular growth in vitro on subsequent oocyte developmental competence

Experiment 2A Follicular Growth and Development.

The gonadotrophin treatment received by the follicles resulted in an alteration to the concentration and production of steroids during the culture period. In order to investigate the effects of this alteration more directly, culture medium was supplemented with an oestrogen agonist, an aromatase inhibitor or both compounds. To elevate oestrogen levels, diethylstilboestrol (DES) was added to culture medium. This compound is structurally related to 17-β-oestradiol and has been used extensively to study reproductive function. In order to elevate androgen levels and lower oestrogen levels, an aromatase inhibitor, Arimidex (Zeneca ZD 1033), was used. This compound selectively blocks the aromatase reaction without inhibiting other steroidal enzymes and without having any direct steroidal actions (Lonning *et al.*, 1998). To elevate levels of both steroids, culture medium was supplemented with both DES and Arimidex. As both DES and Arimidex were solubilised in ethanol before addition to culture medium, control medium was supplemented with the same concentration of ethanol. **Control** follicles were allocated to control medium supplemented with 5% *hpg* serum and 2 IU ml^{-1} FSH. Treatment groups were **High A** (control medium + 0.1 μm Arimidex); **High E** (control medium + 0.004 nmol DES); **High A + E** (control medium + 0.1 μm Arimidex + 0.004 nmol DES).

Follicles were moved daily into fresh wells of medium and cultured for 6 days. At the end of the culture period, representative follicles were labeled for apoptosis using TUNEL. Spent media from all groups were analyzed for steroids.

Elevating oestrogen, androgen or both steroids had no effect on follicular growth or rate of antral development, with all follicles growing well and developing large antral cavities during the culture period, irrespective of treatment (Fig. 5). Similarly, differing steroid treatments had no effect on the degree of apoptotic cell labeling within the follicles (data not shown as these follicles were very similar to those shown in Fig.2, B-D).

In order to check that treatments had successfully altered the level of steroids to which the follicles had been exposed, media samples from day 6 of culture were analyzed for androstenedione and oestradiol. Comparison of the levels of androstenedione in the media showed that the concentration of Arimidex used successfully inhibited aromatase activity. In the High A and High A+E groups, androstenedione levels were significantly raised when compared to the control group or High E group ($p<0.01$, Fig. 6) As expected, inclusion of Arimidex (High A group) also significantly reduced the level of 17-β–oestradiol production by the follicles compared with the mean and s.e.m. of the control group (73.0 ± 19.0 pmol/ml, compared to 229.1 ± 31.7 for the control group; $p<0.001$). We were unable to measure the increase in total oestrogen levels in response to the addition of DES (in both the High E and the High A + E groups), as the oestrogen assay used is highly specific for 17-β-oestradiol and does not cross react significantly with any other oestrogen. There were no detectable steroids in the unincubated culture medium.

Experiment 2B: In Vitro Fertilisation.

In order to minimize any influence of steroids found in serum, CSS was used for these cultures. As the majority of follicles could not maintain their basement membranes on the final two days of culture in CSS, the culture system was modified; on days 1 and 2 of culture, follicles were transferred into wells of fresh medium and damaged or atretic follicles discarded. On day 3 of culture they were then transferred into 60μl of medium overlaid with 75μl of silicone fluid.

Follicles were isolated, placed in the control medium and treatment groups as described in Experiment 2A (i.e. Control, High A, High E and High A + E) and cultured for five days after which COCs were removed from the follicular masses and placed in maturation medium prior to IVF. In all cases medium was supplemented with 1 IU ml^{-1} rhFSH. Three experimental runs were performed with a total of 120 follicles placed in culture for each treatment. The number of available oocytes taken for IVF at the end of the culture period was as follows: Control - 57; High A - 49; High E - 51 and high A + E - 51. As in Experiment 1B, oocytes (50) from superovulated F1 mice were included as a control for the IVF system. Steroid treatment significantly altered the rate of fertilisation ($p< 0.01$). The presence of DES, whether in the High E or the High A + E group, yielded fewer oocytes capable of fertilization when compared to the control group (where steroid production could be expected to follow a more physiological pattern), whereas elevating androgen levels during the culture period yielded a greater percentage of oocytes capable of fertilisation than the control group (Fig. 7). The percentage of two-cell embryos able to complete development to the blastocyst stage was similar irrespective of steroid treatment.

Experiment 3: Effect of alteration in steroid regime during follicular growth in vitro on the global DNA methylation levels of the oocyte.

Experiment 3A: DNA methylation during culture.

Follicles were cultured in medium supplemented with CSS and 1 IU ml^{-1} rhFSH for either 0, 2, 4 or 6 days before they were ruptured and the oocytes collected and fixed. After immunohistochemistry, the DNA methylation pattern and level could be seen to increase with time in culture (Fig. 8): the level of DNA methylation showed a large increase between the start and end of culture, with the staining around the edge of the nucleolus becoming more defined.

Experiment 3B: Global DNA methylation levels after exposure to altered steroidal regime in culture.

Follicles were isolated, placed in the control and steroid treatment groups as described in Experiment 2A (i.e. Control, High A, High E and High A + E) and cultured for 5 days. At the end of the culture period, COCs were removed, oocytes mechanically denuded and used for immunohistochemistry. The experiment was performed a minimum of three times. Differences in global DNA methylation level were evident after steroid treatment (Fig. 9). High A resulted in a significantly lowered methylation level when compared to the controls ($p<0.001$). Whereas when the oocytes were exposed to High A + E, there was an increase in global methylation

over that of the controls ($p < 0.0001$). The High E treatment did not alter methylation level.

Discussion

The results presented here confirm that FSH alone can drive follicular growth and development with the addition of LH having no effect. Other in vitro studies have reported that the inclusion of LH in culture medium accelerates the formation of the antral cavity (Cortvrindt *et al.*, 1998). We found no evidence to support this, as antral formation was similar in all treatment groups, perhaps due to the difference in culture technique. As could be expected, the inclusion of LH in the culture medium augmented the production of androstenedione and oestradiol at an earlier time point in follicular development. Interestingly, FSH alone supported the production of androstenedione, and hence oestradiol, by the follicles, albeit at a later time-point than when LH was also present. We have previously found that under the conditions used in this culture system, follicles exposed to FSH alone produce inhibin (unpublished data) which has been shown to augment androgen synthesis during preovulatory follicle growth (Smyth *et al.*, 1994); this pathway could explain the production of androstenedione (and hence oestradiol) in follicles exposed to FSH alone. Some reports have suggested that high levels of androgens and oestrogens can induce atresia in developing follicles (Billig *et al.*, 1993, Dierschte et al. 1994) while others have shown that these steroids promote follicular development and health (Murray *et al.*, 1998, Vendola *et al.*, 1998). By employing an oestrogen agonist and/or an aromatase inhibitor to alter levels of oestrogens, androgens or both steroids throughout the pre-antral to antral growth phase of follicles, we found that there was no effect on follicular growth, development or health.

While follicular growth and health were unaffected by the different gonadotrophin regimes or steroidal environments used in these experiments, there

was an effect on oocyte developmental competence. There has been some controversy over whether the actions of LH are necessary during follicular development in order to optimize oocyte maturation (Balen *et al.*, 1993, Shoham *et al.*, 1993, Homburg, 1998). Transgenic mice over-expressing LH appear to be fertile and give rise to offspring (Mann *et al.*, 1999). Conversely other studies have indicated that high intra-follicular LH permits the premature resumption of meiosis resulting in 'aged' oocytes (Homburg, 1998) and promotes spontaneous germinal vesicle breakdown (Cortvrindt *et al.*, 1998). The addition of high levels of LH to the culture medium in these experiments did not confer any immediate advantage to the oocyte, as similar fertilisation rates were seen in all the treatment groups. There was, however, a trend suggesting that fertilised oocytes from the high LH group may be less able to proceed to the blastocyst stage ($p=0.08$).

Surprisingly, it was found that around 20% of oocytes obtained from follicles grown in Gn free conditions could fertilise and complete pre-implantation development even though the oocytes were obtained from follicles that had ceased growing by day 3 and exhibited extensive apoptosis in their granulosa cell layers. This low rate of embryo development was similar to that of oocytes from follicles cultured in high levels of LH, despite the fact that the high LH follicles grew well throughout the culture period, with no increased level of atresia. In marked contrast to the oocytes from the Gn free group, oocytes from uncultured follicles of the same starting size were unable to fertilise, demonstrating that even oocytes from the Gn-free group acquired further developmental competence during the culture period. Furthermore, these oocytes must have been relatively unaffected by the level of apoptosis in the somatic cells of the follicles. It was noticeable that the granulosa

cells immediately surrounding the oocytes in the Gn free group tended to be non-apoptotic, perhaps benefiting from the close proximity of the healthy oocyte or perhaps helping to maintain the healthy status of the oocyte.

Under normal physiological conditions, levels of androgen are higher earlier in follicular development while higher oestrogen levels become more prevalent as follicles progress towards ovulation (Moor *et al.*, 1998). Disruption of this pattern has been correlated with poor fertilisation and development rates (Andriesz and Trounson, 1995, Xia and Younglai, 2000). We have shown here that altering the concentration of steroids in vitro affected the percentage of oocytes undergoing fertilisation and cleavage to the two-cell stage, with fewer oocytes developing to the two-cell stage under conditions of high levels of oestrogen in the form of DES. DES is structurally related to 17-β-oestradiol and is equally (if not more) potent, with a longer half life. In addition, it has been shown to bind to both oestrogen receptors with a higher potency than oestradiol (Guttendorf and Westendorf, 2001). While high levels of LH stimulated steroid production earlier in the culture period, the effect was relatively minor and probably not comparable to adding DES throughout the culture period. This might explain why the LH treatment did not significantly affect fertilisation or embryo development. There is little information on the effects of elevated oestrogen concentrations during follicular development on subsequent oocyte developmental competence. Studies from clinical situations, where supraphysiological oestrogen levels often result as a consequence of ovarian hyperstimulation, are difficult to interpret as any effects seen may be a result of poor oocyte quality or the effects of elevated oestrogen on other areas of the reproductive tract. By using an individual follicle culture system, this current study has

demonstrated for the first time that elevation of oestrogen during the pre-antral to antral growth phase of the follicle is detrimental to the ability of the oocyte to undergo fertilisation.

Oestrogen has been implicated in increasing free calcium stores within oocytes (Tesarik and Mendoza, 1995) and ensuring genomic integrity of oocytes before ovulation (Murdoch and Van Kirk, 2001). In primates, including humans, it has been suggested that lack of oestrogen results in oocytes less able to undergo fertilisation (Balasch *et al.*, 1995) and it has been demonstrated that the addition of steroids during in vitro maturation can aid oocyte developmental competence (Zheng *et al.*, 2003). In contrast, studies from transgenic mouse models that lack either of the oestrogen receptors or that lack the aromatase gene have indicated that oestrogen is not obligatory for follicular development or oocyte maturation (Couse *et al.*, 1999, Huynh *et al.*, 2004). It is also perhaps relevant that the higher fertilisation rate of oocytes obtained from Arimidex-treated follicles described here was the result not only of high levels of androgen but also of lowered oestrogen levels, showing that decreased oestrogen levels cannot have been detrimental. A recent study utilizing Arimidex to inhibit aromatase activity in vivo concluded that rising oestrogen levels were not a requirement for either follicular development or oocyte maturation (Guo *et al.*, 2004).

We have shown that oocytes lay down increased levels of DNA methylation during the culture period. Similar increases in global DNA methylation levels over the equivalent stages of follicle growth in vivo have been observed (unpublished observations, Swales & Spears) and increases in the methylation level of specific sequences have been shown to occur over oocyte growth in vivo (Lucifero *et al.*,

2004). The steroidal environment affected oocyte global DNA methylation levels: the oocytes of follicles cultured in high levels of androgens and oestrogens had raised DNA methylation levels, while those from follicles cultured in high levels of androgen alone had reduced DNA methylation levels. DNA methylation of oocytes thus showed an inverse correlation to the fertilisation data obtained: oocytes from the high A group, which had lowered DNA methylation levels, had the best fertilisation rate while those from the high A + E group, with increased DNA methylation levels, had a significantly reduced fertilisation rate, compared to that of controls. It is possible that inappropriate DNA methylation is one of the causes of poor fertilisation rates. DNA methylation is part of the mechanism involved in controlling normal expression patterns of imprinted genes: the correct establishment of genomic imprinting during oocyte growth and maturation is vital if normal fertilisation and embryo development are to occur (Obata *et al.*, 1998). Additionally, DNA methylation is implicated in preventing the movement of retro-transposons and p53-dependent apoptosis (Dean *et al.*, 2001, Jackson-Grusby *et al.*, 2001). It cannot be determined from the experiment described here whether the changes in DNA methylation are due to alterations in levels of imprinted genes, other regions of the genome or a combination of both. As the observed alterations in DNA methylation were quite large, it is probably unlikely that these changes would be due only to changes in imprinted genes, as the DNA methylation associated with these imprinted genes is relatively small when compared to that of the non-imprinted genomic regions. Alterations to global DNA methylation levels are associated with failure of cloned embryos (Dean *et al.*, 2001), while errors in the DNA methylation of specific

imprinted genes can result in disorders or disease in subsequent offspring (Mizuno *et al.*, 2001, Young *et al.*, 2001)

Manipulating the ovary, such as through the administration of exogenous gonadotrophins, can alter the steroidal environment within those follicles and subsequently have an effect on the developmental competence of the oocyte. While the idea that steroids influence oocyte competence is not new, these present studies have shown for the first time that inappropriate exposure of the oocyte to steroids during follicle maturation may be detrimental to oocyte developmental competence and impact upon DNA methylation of the genome.

Acknowledgements

The authors wish to thank Vivian Allison for expert assistance with the follicle cultures and Martha Urquhart for performing the androstenedione assays. Dr Vlastimil Sršeň kindly carried out the TUNEL staining of the follicles and Linda Wilson performed the confocal microscopy. Dr Nathalie Beaujean kindly advised on the methylation immunohistochemistry protocols.

Grant Support. Supported by MRC grant G9901514. AKES supported by BBSRC.

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Figure Legends.

Figure 1. Growth rates of follicles exposed to different gonadotrophin environments. Values are mean ± SEM (n ≥16 for each group). Arrow indicates the start of antral formation iin all gonadotrophin groups.

Figure 2. Confocal micrographs after labeling with TUNEL (green) and propidium iodide (red). Green cells indicate apoptosis. Representative follicles are shown for (**A**) Gn Free, (**B**) FSH only, (**C**) low LH, (**D**) high LH. The follicle grown in (**A**) Gn free conditions shows a high degree of apoptotic labeling. Scale bar represents 50μm.

Figure 3. Concentration of (**A**) androstenedione and (**B**) 17-β -estradiol produced by follicles treated with different gonadotrophin regimes. Media were sampled on days 4 and 5 of culture. Values are mean ± SEM (n ≥ 9 for each group). Different superscripts indicate significant differences. a,b refer to comparisons between treatments on the same day of culture and x,y refer to comparisons between days within the same treatment ($p<0.05$).

Figure 4. Percentage of oocytes from follicles grown in different gonadotrophin conditions (**A**) fertilising and reaching the 2-cell stage and (**B**) 2-cell embryos developing to the blastocyst stage. F1 controls are shown for comparison.

Figure 5. Growth rates of follicles cultured in control medium, High A, High E and High A + E treatment groups. Values are mean ± SEM (n ≥16 for each group). The arrow indicates the start of antral formation in all groups.

Figure 6. Concentration of androstenedione produced by follicles grown in different steroid environments. Media were sampled on day 6 of culture. Values are mean ± SEM (n ≥ 9 for each group). * $p<0.001$ compared to control.

Figure 7. Percentage of oocytes from follicles grown in different steroid environments (**A**) fertilising and reaching the 2-cell stage and (**B**) 2-cell embryos developing to the blastocyst stage. F1 controls are shown for comparison.

Figure 8. Confocal images of global DNA methylation levels of oocytes removed from culture on Days 0, 2, 4 and 6 and stained using a 5-methyl-cytosine antibody and FIT-C secondary. Scale bars represent 10μm.

Figure 9. Normalized global DNA methylation levels for each oocyte according to treatment group, Control, High A, High E and High A + E. Horizontal bars represent the mean of the treatment group (n ≥48 for each group).

Fig 1

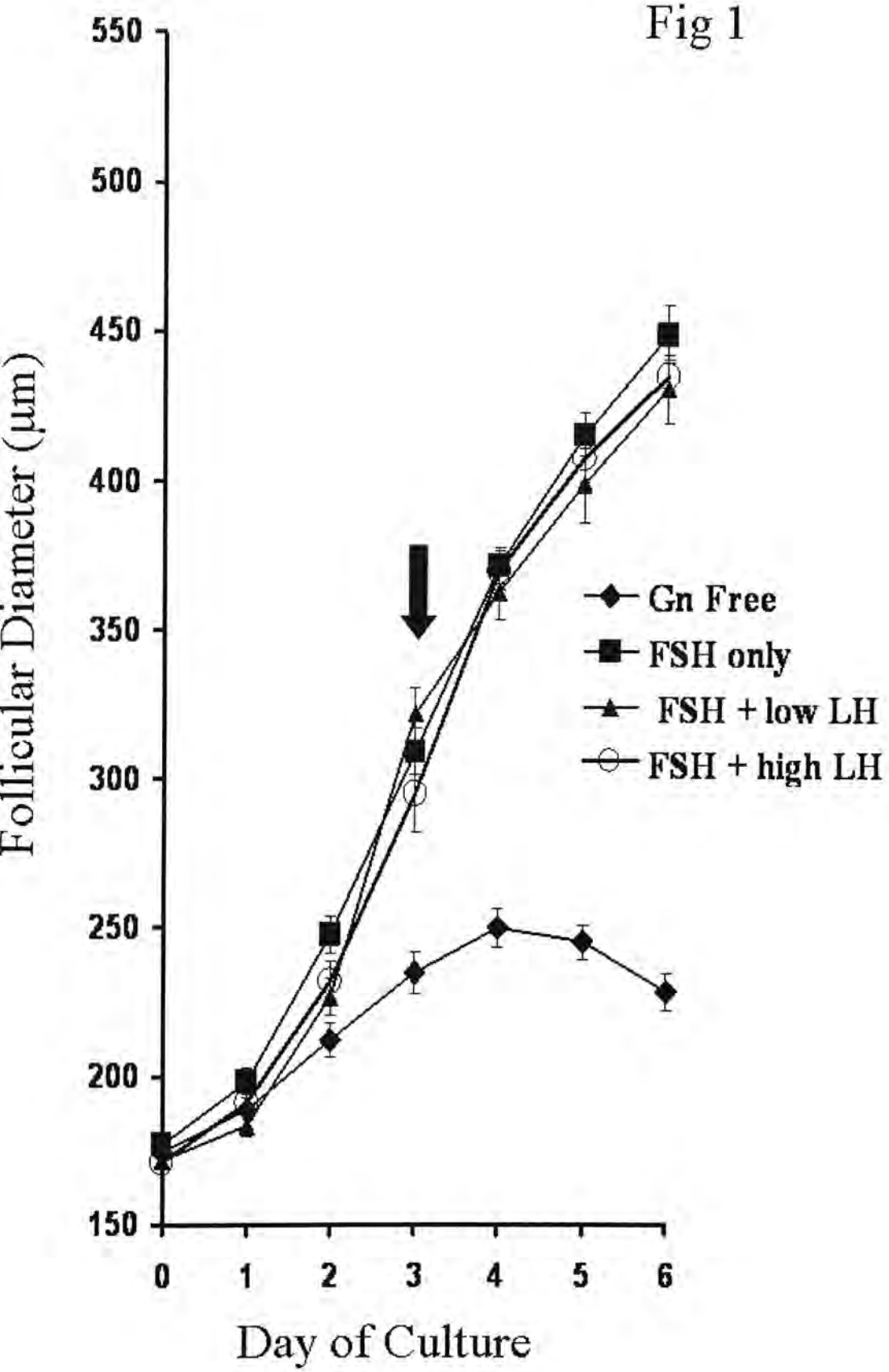

550
500
450
400
350
300
250
200
150
Follicular Diameter (µm)
0
1
2
3
4
5
6
Day of Culture
Gn Free
FSH only
FSH + low LH
FSH + high LH

Fig 2

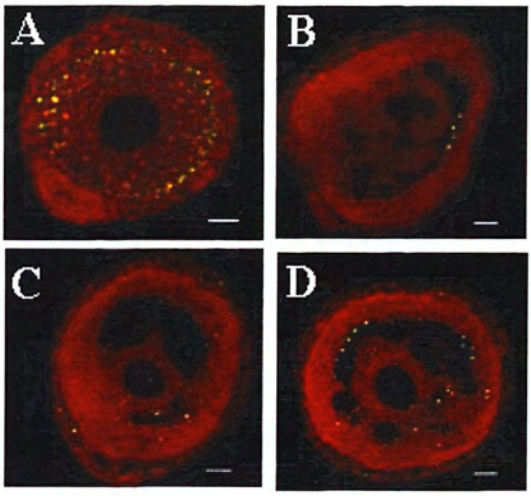


Fig 8

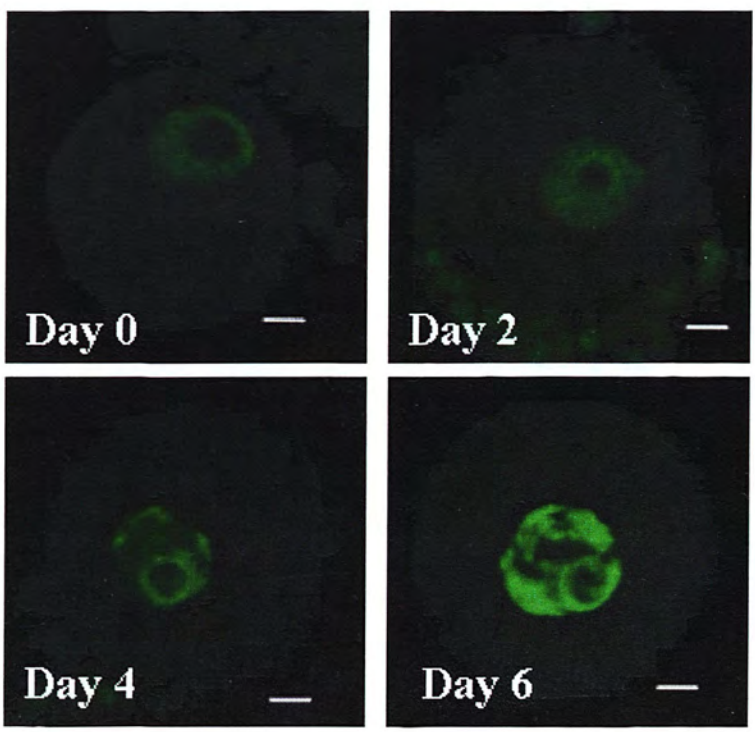


Fig 3

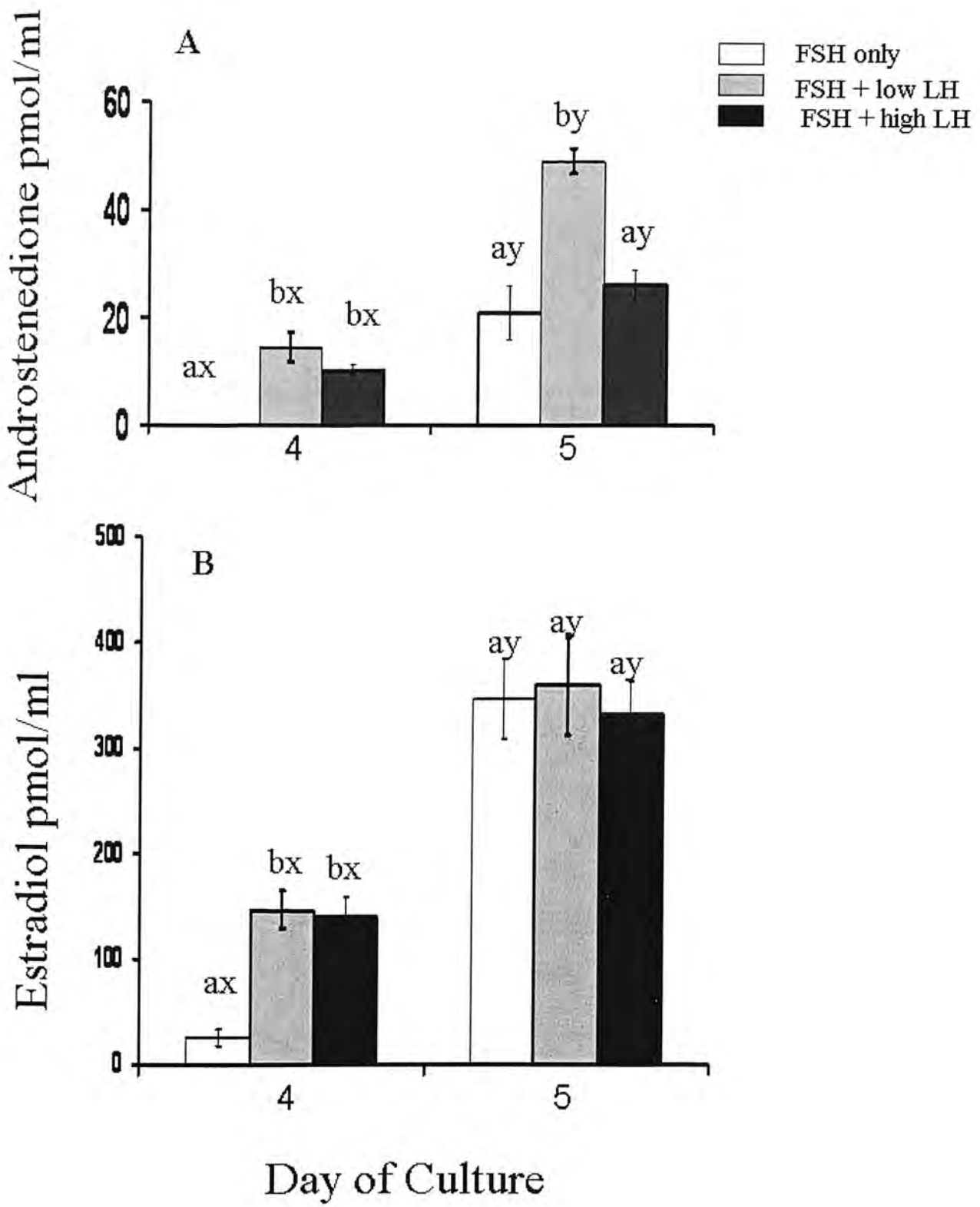

A
FSH only
FSH + low LH
FSH + high LH
Androstenedione pmol/ml
60
40
20
0
ax
bx
bx
ay
by
ay
4
5
B
Estradiol pmol/ml
500
400
300
200
100
0
ax
bx
bx
ay
ay
ay
4
5
Day of Culture

Fig 4

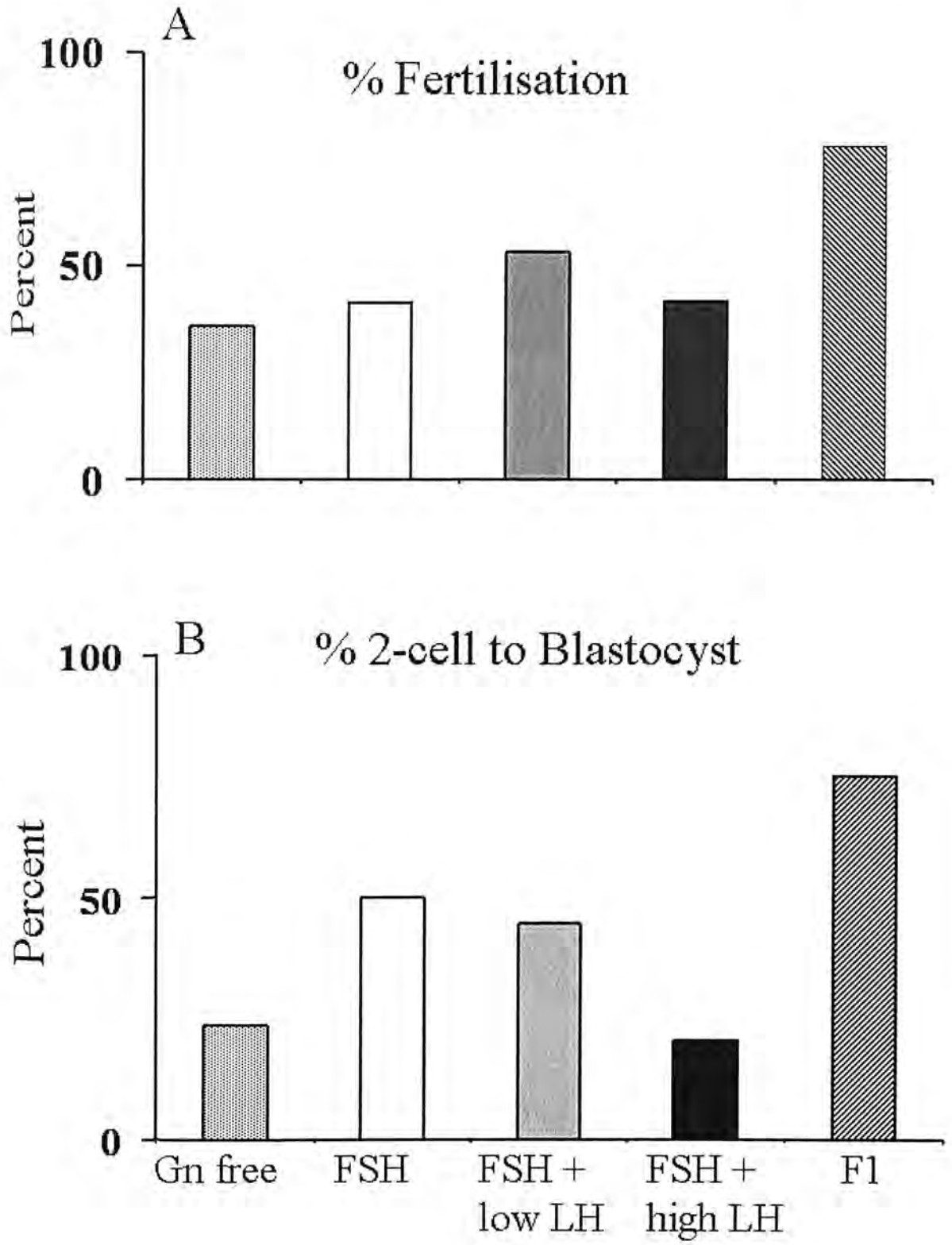

A
% Fertilisation
100
50
0
Percent
B
% 2-cell to Blastocyst
100
50
0
Percent
Gn free
FSH
FSH + low LH
FSH + high LH
F1

Fig 5.

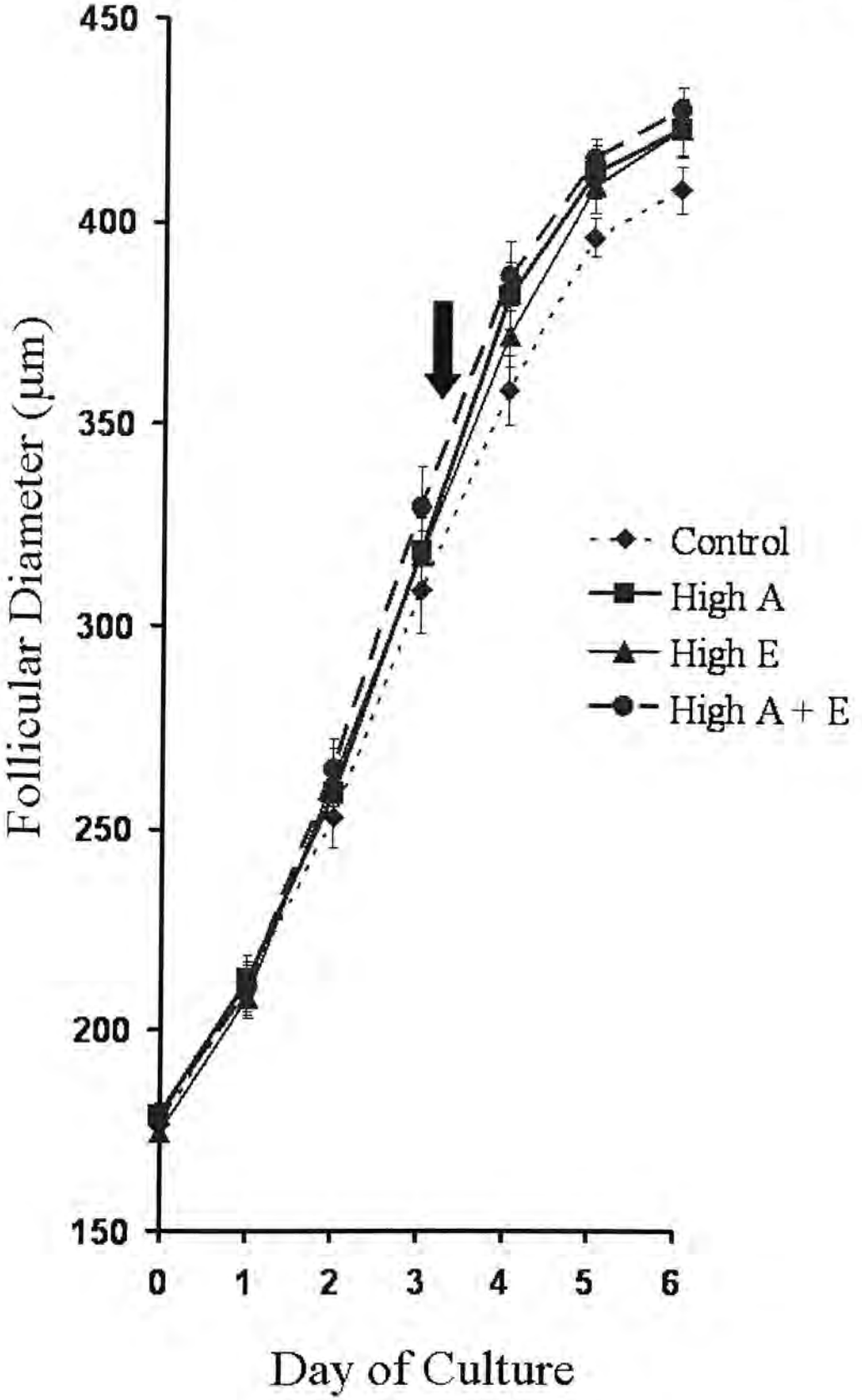

450
400
350
300
250
200
150
Follicular Diameter (µm)
0
1
2
3
4
5
6
Day of Culture
Control
High A
High E
High A + E

Fig 6.

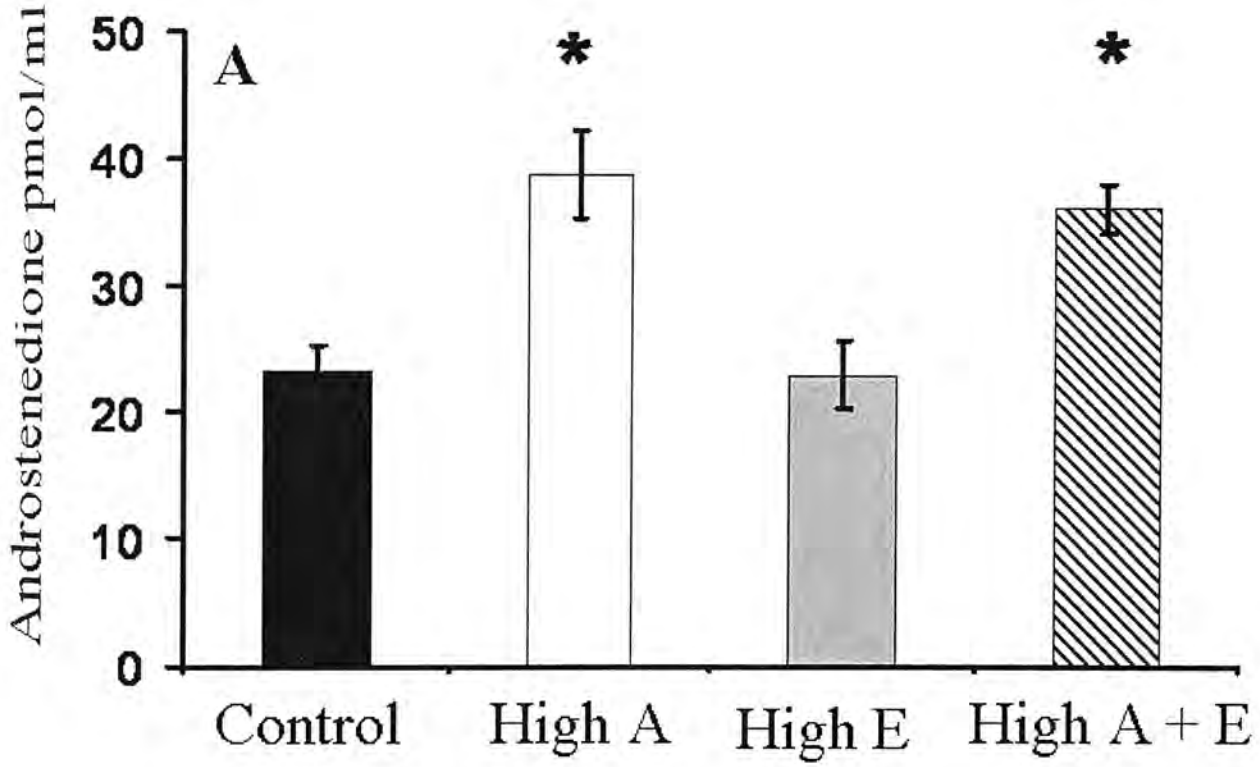

Androstenedione pmol/ml
50
40
30
20
10
0
A
*
*
Control
High A
High E
High A + E

Fig 7

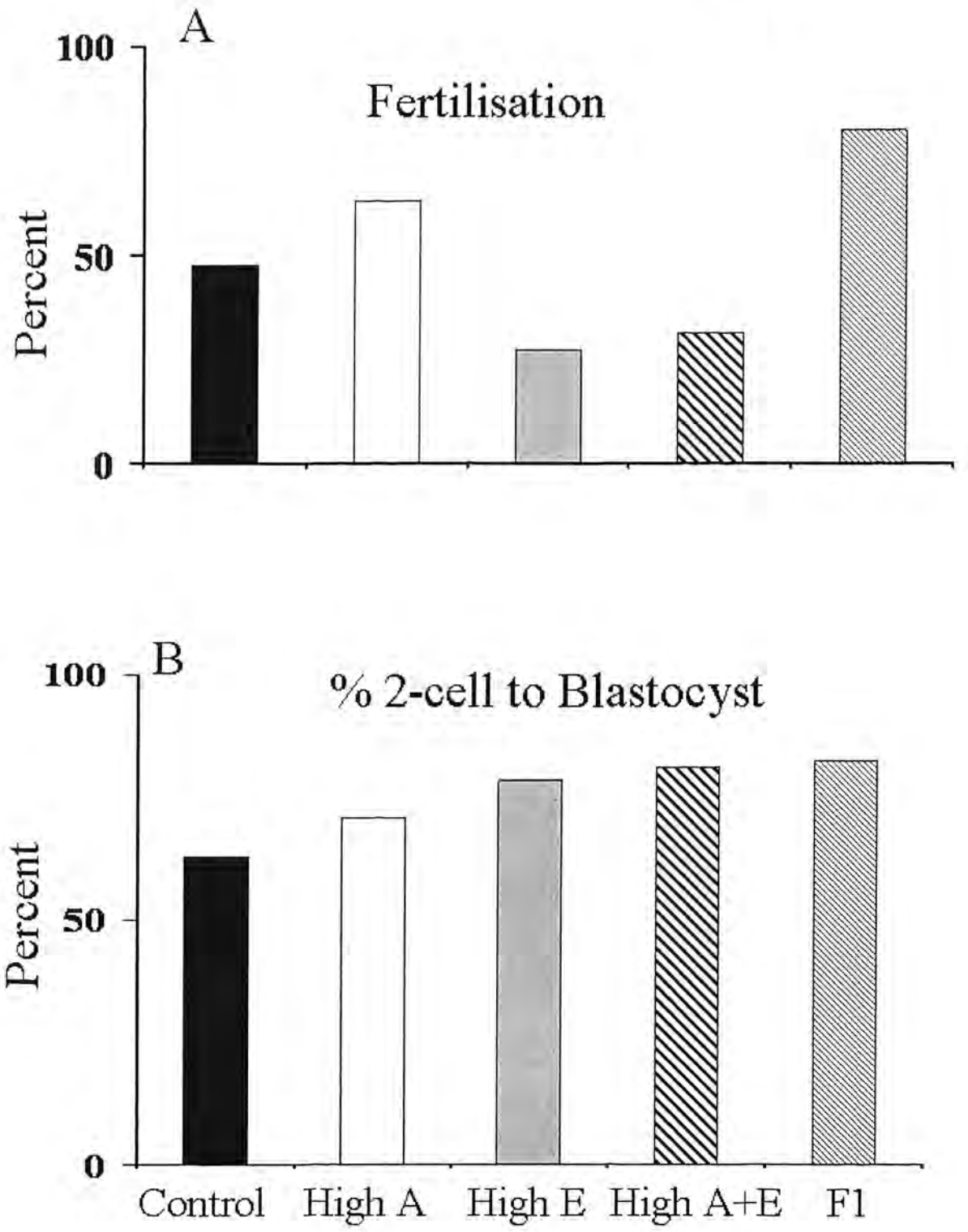
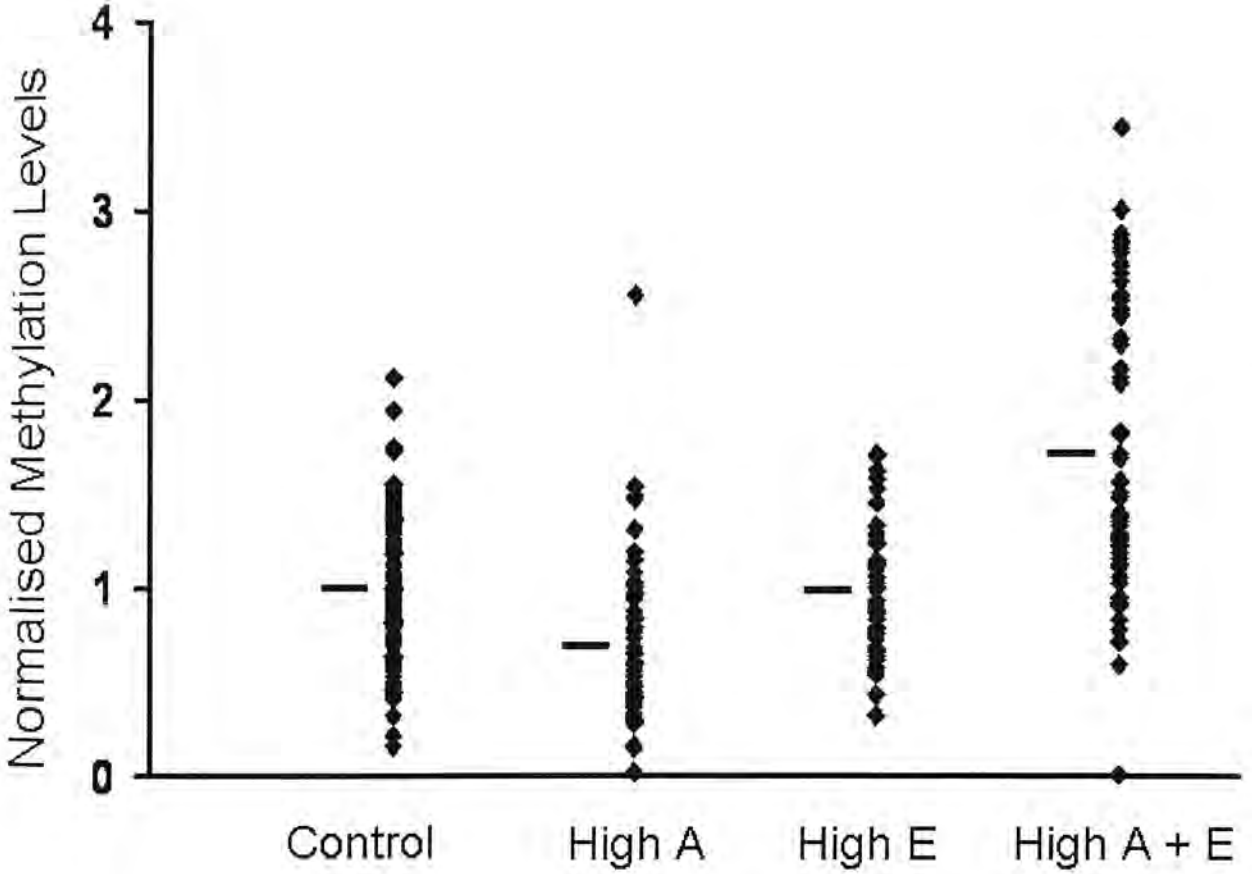

A
Fertilisation
Percent
100
50
0
B
% 2-cell to Blastocyst
Percent
100
50
0
Control
High A
High E
High A+E
F1

Fig 9


Normalised Methylation Levels
4
3
2
1
0
Control
High A
High E
High A + E